MOLECULAR EPIDEMIOLOGY OF RESPIRATORY VIRUSES IN A PAEDIATRIC COHORT FROM INDONESIA

SHIRLENA SOH WEE LING
B Sc Hons *(University of Tasmania),*
M Sc *(National University of Singapore)*

Cell and Molecular Biosciences
FACULTY OF SCIENCE AND TECHNOLOGY, QUT
AND
INSTITUTE OF HEALTH AND BIOMEDICAL INNOVATION, QUT
AND
GENOME INSTITUTE OF SINGAPORE (GIS)

A thesis submitted for the degree of Doctor of Philosophy at
Queensland University of Technology

2010

ABSTRACT

Acute lower respiratory tract infections (ALRTIs) are a common cause of morbidity and mortality among children under 5 years of age and are found worldwide, with pneumonia as the most severe manifestation. Although the incidence of severe disease varies both between individuals and countries, there is still no clear understanding of what causes this variation. Studies of community-acquired pneumonia (CAP) have traditionally not focused on viral causes of disease due to a paucity of diagnostic tools. However, with the emergence of molecular techniques, it is now known that viruses outnumber bacteria as the etiological agents of childhood CAP, especially in children under 2 years of age.

The main objective of this study was to investigate viruses contributing to disease severity in cases of childhood ALRTI, using a two year cohort study following 2014 infants and children enrolled in Bandung, Indonesia. A total of 352 nasopharyngeal washes collected from 256 paediatric ALRTI patients were used for analysis. A subset of samples was screened using a novel microarray pathogen detection method that identified respiratory syncytial virus (RSV), human metapneumovirus (hMPV) and human rhinovirus (HRV) in the samples. Real-time RT-PCR was used both for confirming and quantifying viruses found in the nasopharyngeal samples. Viral copy numbers were determined and normalised to the numbers of human cells collected with

the use of 18S rRNA. Molecular epidemiology was performed for RSV A and hMPV using sequences to the glycoprotein gene and nucleoprotein gene respectively, to determine genotypes circulating in this Indonesian paediatric cohort.

This study found that HRV (119/352; 33.8%) was the most common virus detected as the cause of respiratory tract infections in this cohort, followed by the viral pathogens RSV A (73/352; 20.7%), hMPV (30/352; 8.5%) and RSV B (12/352; 3.4%). Co-infections of more than two viruses were detected in 31 episodes (defined as an infection which occurred more than two weeks apart), accounting for 8.8% of the 352 samples tested or 15.4% of the 201 episodes with at least one virus detected. RSV A genotypes circulating in this population were predominantly GA2, GA5 and GA7, while hMPV genotypes circulating were mainly A2a (27/30; 90.0%), B2 (2/30; 6.7%) and A1 (1/30; 3.3%).

This study found no evidence of disease severity associated either with a specific virus or viral strain, or with viral load. However, this study did find a significant association with co-infection of RSV A and HRV with severe disease ($P = 0.006$), suggesting that this may be a novel cause of severe disease.

LIST OF PUBLICATIONS

SOH, S., AW, P., SOH, H., KARTASAMITA, C., SIMOES, E. & HIBBERD, M. 2009. Infection with both human rhinovirus and respiratory syncytial virus A is associated with increased disease severity in children. *In* Abstracts of *Genetics and Genomics of Infectious Diseases* (March 21-24), Singapore

WONG, C., HENG, C., WAN YEE, L., SOH, S., KARTASASMITA, C., SIMOES, E., HIBBERD, M., SUNG, W. & MILLER, L. 2007. Optimization and clinical validation of a pathogen detection microarray. *Genome Biol,* 8**,** R93.

SIMOES, E., MUTYARA, K., SOH, S., AUGUSTIAN, D., HIBBERD, M., KARTASASMITA, B. The Epidemiology of Respiratory Syncytial Virus Lower Respiratory Tract Infections In Under Fives In Indonesia (to be submitted to American Journal of Respiratory and Critical Care Medicine for publication)

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ALTRIs	acute lower respiratory infections
ARTIs	acute respiratory tract infections
bp	base pair(s)
CAP	community acquired pneumonia
CDC	Centers for Disease Control and Prevention
cDNA	complimentary DNA
COPD	chronic obstructive pulmonary disease
Ct	cycle threshold
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbent assay
FAM	6-carboxy-fluorescein
GIS	Genome Institute of Singapore
HD	probe hamming distance
HEX	hexachloro-6-carboxy-fluorescein
hMPV	human metapneumovirus
HRV	human rhinoviruses
ICAM	intercellular adhesion molecule
kb	kilobase
LRTIs	lower respiratory tract infections

MCM	maximum contiguous match
µL	microliter
µM	micromolar
mL	millilitre
mM	millimolar
mRNA	messenger RNA
NCBI	National Center for Biotechnology Information
ng	nanogram
NJ	neighbour-joining
nm	nanometer
nM	nanomolar
nt	nucleotide
PCR	polymerase chain reaction
RNA	ribonucleic acid
RR	relative risk
RSV	respiratory syncytial virus
RT-PCR	reverse transcriptase PCR
TAMRA	6-carboxy-tetramethyl-rhodamine
TBE	Tris-Boric Acid
U	unit
URT	upper respiratory tract
WHO	World Health Organisation

LIST OF APPENDICES

STATEMENT OF ORIGINAL AUTHORSHIP

The work contained in this thesis has not been previously submitted for a degree or diploma at this or any other higher education institution. To the best of my knowledge and belief, the thesis contains no material previously published or written by any other person except where due reference is made.

Signed: 

Shirlena Wee Ling Soh, BSc, MSc

Date: 1st June 2010

STATEMENT OF CONTRIBUTION OF THE CANDIDATE

Clinical Samples: Upon the arrival of clinical samples from Bandung, Indonesia, Shirlena Soh was in-charge of coordinating and liaising with the Indonesia and the United States counter parts on the clinical databases of the patients, which included appraising of the clinical data and checking its validity. She was also involved in the extraction of both RNAs and DNAs from the nasopharyneal washes.

Microarray Assays: Shirlena Soh with the guidance of her local supervisor, A/Prof Martin Hibberd, worked and liaised with the Microarray team in Genome Institute of Technology, Singapore on the selection of pathogens for the pathogen chip design. She also co-performed both the experimental work and the analysis of the microarray data obtained.

PCR Assays: Shirlena Soh with the guidance of A/Prof Martin Hibberd conceived the experimental design of the work, designed the primers and probes for hMPV, performed the PCR and cloning works, evaluated and analysed the data obtained. She was also directly involved in the statistical analyses of all data obtained including the meteorological data with the assistance of a statistician, Harold Soh. In the year 2005, Shirlena Soh and her supervisor were among the first to attempt to correlate viral loads to disease severity in RSV, hMPV and HRV.

Sequencing and Phylogenetic Analyses: Shirlena Soh conceived the experimental design of the work, evaluated and designed the primers and probes for RSV A and hMPV sequencings, performed phylogenetic analyses on the sequences and analysed the data obtained.

STATEMENT OF CONTRIBUTIONS TO JOINTLY AUTHORED WORKS CONTAINED IN THIS THESIS

SOH, S., AW, P., SOH, H., KARTASAMITA, C., SIMOES, E. & HIBBERD, M. 2009. Infection with both human rhinovirus and respiratory syncytial virus A is associated with increased disease severity in children. *In* Abstracts of *Genetics and Genomics of Infectious Diseases* (March 21-24), Singapore:

Shirlena Soh conceived, wrote and presented the work.

WONG, C., HENG, C., WAN YEE, L., SOH, S., KARTASASMITA, C., SIMOES, E., HIBBERD, M., SUNG, W. & MILLER, L. 2007. Optimization and clinical validation of a pathogen detection microarray. *Genome Biol,* 8, R93:

Shirlena Soh designed the primers for hMPV, performed the real-time RT-PCR (RSV A, RSV B, hMPV and RSV), assisted in the evaluation and analysis of the data obtained.

STATEMENT OF CONTRIBUTION BY OTHERS

TO THE THESIS AS A WHOLE

Eric Simoes (University of Colorado at Denver Health Sciences Center and The Children's Hospital, USA), Cissy Kartasasmita and team (Padjadjaran University/Hasan Sadikin General Hospital, Java Indonesia) provided nasopharyngeal washes and clinical data for patients in the study.

Christopher Wong and team (Genome Institute of Technology, Singapore) provided the pathogen chip for screening of nasopharyngeal aspirate samples. They co-performed the experimental work for the microarray and assisted in the analysis of the microarray data obtained.

Pauline Aw (Genome Institute of Technology, Singapore) assisted in RNA extraction from nasopharyngeal samples and sequencing of hMPV and RSV A.

Harold Soh (Institute of High Performance Computing, Singapore) assisted in the statistical analysis of weather data.

Michael Edwards (Michael.edwards@imperical.ac.uk) provided sequences and clone for the detection of human rhinovirus.

Bernadette van den Hoogen and team (Erasmus Medical Center Rotterdam, The Netherlands) provided clones for human metapneumovirus.

ACKNOWLEDGEMENTS

I wish to express my sincere thanks and appreciation to my supervisor, Assistant Professor Martin L. Hibberd, for giving me this opportunity to work with him and his wonderful team. I am most grateful for his constant guidance, his patience and his understanding of my work.

I am also grateful to my other supervisors Associate Professor Louise Hafner and Professor Peter Timms for their encouragement and for always being there when I needed them.

Pauline Aw, my most appreciated work mate. Without you, this will have been a much tougher quest to conquer. Harold Soh, one of the most brilliant persons that I have met, thank you for all the complicated statistical work. To Ling Ling, YuQing and Wee Yang for their assistances one way or another.

I acknowledge with thanks the Queensland University of Technology (QUT) and the Faculty of Science and Technology for funding my QIDS scholarship and the Genome Institute of Singapore for funding my research.

Lastly and mostly to Gary and Kayser, my inspirations, the reasons that make this quest so much more bearable and without them, this would not have been possible.

CHAPTER 1 – LITERATURE REVIEW

1.1 Paediatric Respiratory Tract Infections

Acute respiratory tract infections (ARTIs) are a common cause of morbidity and mortality among children (Gesteland *et al.,* 2007; Zalm *et al.,* 2008, Sung *et al.,* 2009). ARTIs have been reported as being among the leading causes of death in children aged less than 5 years worldwide (Nascimento-Carvalho *et al.,* 2001; Gesteland *et al.,* 2007; Mackay, 2008; www.who.int). Infections of the lower respiratory tract are common in the community and comprise both acute bronchitis, acute exacerbations of chronic bronchitis, acute bronchiolitis and pneumonia (Feldman *et al.,* 2003; Barben *et al.,* 2008; Mahony, 2008; Miron *et al*., 2010; www.who.int).

Acute bronchitis has been described as a recent onset of cough, with sputum production, in a patient with no evidence of underlying chronic obstructive pulmonary disease (COPD), sinusitis or pneumonia. It is a common condition largely diagnosed and managed in general practice. Most cases, particularly those occurring in previously healthy individuals, are presumed to be viral in origin and very low rates of bacterial isolation have been noted (Feldman, 2004; Okiro *et al.,* 2008; Kim *et al.,* 2009).

Bronchiolitis is an acute, infectious, inflammatory disease of the upper and lower respiratory tract that may result in obstruction of the small airways and is one of the most common acute lower respiratory diseases in young children and the

leading cause of hospitalization, during the winter, worldwide (Livni *et al*., 2010; Miron *et al*., 2010). Although it may occur in all age groups, the larger airways of older children and adults better accommodate mucosal oedema; severe respiratory symptoms are usually limited to young infants. The severity of the disease is usually related to the degree of inflammation of the small bronchioles, however, acute bronchiolitis can also involve the upper airways, causing nasal congestion, inadequate oral intake, dehydration, respiratory distress and, sometime hypoxemia (Livni *et al.*, 2010).

Pneumonia, the most severe form of acute lower respiratory tract infections (ALRTs) is a serious infection or inflammation of the lungs and can occur in several forms. Lobar pneumonia is limited to a section (or lobe) of a lung, whereas bronchial pneumonia (or bronchopneumonia) affects patches throughout the lungs. The World Health Organization (WHO), as part of their Integrated Management of Childhood Illness program, recommends diagnosis of pneumonia using clinical signs such as tachypnea (respiratory rate greater than 50/min in infants younger than 1 year of age and greater than 40/min in children older than 1 year of age), retractions, or cyanosis (WHO, 1995; Lichenstein *et al.*, 2003).

Pneumonia is not a single disease but a description of a clinical syndrome and the disease has over 30 causes ranging from bacteria, viruses, mycoplasma and other non-infectious substances (Puli *et al.*, 2002). Bacterial organisms

are largely responsible for most ALRTI deaths occurring in the developing world. Even though mortality is very much lower with viral pneumonia (Nascimento-Carvalho, 2001; Smyth, 2002), complications may arise and death by secondary bacterial infections can result (Farha *et al.*, 2005). Deaths from pneumonia are rare in developed countries; it however was responsible for between 3 – 18% of all paediatric admissions (Farha *et al.*, 2005). In the developing countries, particularly in Asia and Africa, death from pneumonia is estimated at 3 million annually (Singh, 2005).

1.2 Etiological Agent of Pneumonia

Almost all acute respiratory tract infection-related deaths were in children with pneumonia. However, determining the etiologic agent of pneumonia is a difficult diagnostic problem since appropriate specimens can rarely can be obtained from the lower respiratory tract. This is coupled with the fact that many pathogens causing the disease can only be detected by the specific and limited methods available in research laboratories (Murdoch, 2004). In severe cases of pneumonia, shedding of the causative pathogens in other body fluids such as blood, urine and pleural fluid is possible (Coote *et al.*, 2000). Depending upon the number of tests used to detect the pathogen in for example, nasopharyngeal aspirates, the potential causative agent of the disease has been identified in 24 to 85% of cases, leaving a large cohort of patients with unidentified agents of infection (Nascimento-Carvalho, 2001; Sinaniotis, 2004). Identification of pathogens causing ALRTIs helps to determine the clinical management of patients and thus it is extremely important to develop better techniques to improve the detection of potential pathogens.

Community-acquired pneumonia is one of the most common serious infections in children and depending on the regions, the incidence of pneumonia per 100 children ranges from 3.0% to 3.6% in the United States; 18% in Africa and 7% to 40% in the Asia Pacific countries (Nascimento-

Carvalho, 2001). In another report, Singh (2005) noted that pneumonia can cause around 2 million children's deaths annually (20% of all child deaths; www.who.int) with 70% of all deaths recorded in Africa and Asia. When diagnosing community-acquired pneumonia, physicians rely on patient's history and physical examination, supplemented by judicious use of chest radiographs and laboratory tests as needed. The child's age is also an important factor when making the diagnosis. Pneumonia in neonates younger than three weeks of age most often is caused by an infection obtained from the mother at birth (Ostapchuk *et al.*, 2004).

The symptoms of viral pneumonia include fever, cough, headache, and muscle pain and weakness. Symptoms are often described as "flu like". Usually, within one or two days of infection, increasing breathlessness develops, the initial cough becomes worse and small amounts of mucus are produced and are accompanied by a high fever. Subsequently, viral pneumonia may be complicated by bacterial infection (Chien *et al*, 2000).

Bacterial pneumonia can affect individuals of any age, from infants through to the very old. Alcoholics, the debilitated, post-operative patients, people with other respiratory diseases or viral infections and those who have a weakened immune system are at greater risk. Bacteria that cause pneumonia may already be carried in the throats of healthy individuals. However, when body defences are weakened, bacteria can multiply and cause disease by entering the lungs

and causing inflammation of the alveoli and the lobes of the lungs and can develop into consolidation with swelling and hardening of the normally soft tissues. Infection can spread quickly and septicaemia may set in. For most of the severe cases, symptoms of patients includes chills, severe chest pain and a productive cough with raised body temperature to over 40◦C. Difficulties in breathing accompanied with raised pulse rate may be seen and patients may also be confused and delirious, especially in adults (Puli *et al.,* 2002; Lichenstein, 2003).

Many studies conducted on childhood community-acquired pneumonia in the developed world have identified a proportion of patients in whom both a virus and a bacterium or two bacteria or two viruses have been identified. These mixed infections accounted for about 10 to 40% of the overall (Farha *et al.*, 2005). It is likely that co-infection is a significant factor in severity and may be much more frequent in hospitalized children. For example, the role of influenza infection in severe pneumococcal pneumonia was highlighted in a study conducted by O'Brien *et al.*, (2004).

1.2.1 Epidemiology of Bacterial Pneumonia

In the developed world, the predominant pathogen in community acquired pneumonia is *Streptococcus pneumoniae* (pneumococcus), an organism that

accounts for about two-thirds of bacterial pneumonia and is the most common cause of bacterial pneumonia in infants three weeks to three months of age (Ostapchuk *et al.*, 2004). Other causative agents of bacterial pneumonia include *Haemophilus influenzae*, *Moraxella catarrahalis*, *Mycoplasma pneumoniae* and *Chlamydia pneumoniae* (Nascimento-Carvalho, 2001).

Currently, there are more than 90 different pneumococcal serotypes or subgroups, and a portion of approximately 20 serotypes are highly invasive and account for most diseases. Pneumococci with reduced susceptibilities to penicillin can be found in many parts of the world, but with variable prevalence (Wolf *et al.*, 2000). In most of the reported studies to date, pneumococci of serogroups 6, 9, 14, 19 and 23 were most frequently associated with penicillin and multidrug resistance (Overweg *et al.*, 1999: Wolf *et al.*, 2000). In a recent study conducted in the United States, Schrag *et al.*, (2004) noted the emergence of *S. pneumoniae* with very high level resistance to penicillin (MIC ≥ 8ug/ml). The multiresistant type 23F clone (Spain 23F-1) had been prevalent in Spain for more than 20 years. This clone has since spread worldwide, and predominated in countries such as in the European countries, South Africa and the United States (Dowson *et al.*, 1994; Ferroni *et al.*, 1996). Most of the penicillin-resistant *S. pneumoniae* strains isolated in Asia were found to be those of 19 or 23F and among the Asian 23F clones, some were indistinguishable from the Spanish serotype 23F clone while others were variants of the Spanish clone (Soh *et al.*, 2000). In the late

1980s, a type 6B clone (Spain 6B-2) imported from Spain caused a rapid increase in the prevalence of penicillin resistance among pneumococcal strains in the UK and Iceland (Dowson *et al.*, 1994). A third, extensively spread clone, sometimes referred to as the "French/Spanish clone", usually classified as serotype 9V was found to have spread to several countries in Europe and South America (Gasc *et al.*, 1995). Although the spread of these clones is constantly a major cause of concern, it is also heartening to note that, with the introduction of the pneumococcal conjugate vaccine and with proper treatment, the incidence of these resistant clone infections can be decreased by as much as 50% among children < 5 years of age, in particularly the serotypes 23F and 14 (Schrag *et al.*, 2004).

Mycoplasma pneumoniae and *Chlamydia pneumoniae* often are the etiologic agents of pneumonia in children older than five years and in adolescents (Lichenstein *et al.*, 2003). Owing to the somewhat different symptoms and physical signs of disease, *Mycoplasma* and *Chlamydia pneumoniae* were once believed to be caused by one or more undiscovered viruses, and were known as primary atypical pneumonia. A most prominent symptom of these pneumonia is a cough that tends to come in violent attacks but produces only sparse mucus. Chills and fever are early symptoms and some individuals may experience nausea and vomiting. The death rate is low, even in untreated cases. In the most recent study done in USA, atypical pneumonia caused by *M. pneumoniae* accounted for 14% of cases where a pathogen was identified and

C. pneumoniae in 9% of cases. In contrast with other studies, it was found that preschool-aged children had as many episodes of atypical bacterial lower respiratory infections as older children (Michelow *et al.,* 2004: Farha *et al.,* 2005).

It is difficult to investigate the causative agents for bacterial pneumonia, as several things can interfere with diagnosis of the infection. One is the establishment of a true infection versus colonization of a potential pathogen and this problem cannot be overcome by routine bacterial culture performed in the clinical microbiology laboratory. Secondly, unexpected common Gram-negative bacteria isolated in respiratory cultures may be overlooked and be mistaken as contaminants or as a commensals (Saiman, 2004). In addition, many research studies are now reporting co-infections of bacteria and viruses in the respiratory tract and these cases are most of the time missed in the routine testing as microbiologists, based on the patient's clinical symptoms, usually only look for one causative agent (Korppi *et al.,* 1993; Farha *et al.,* 2005). It is also not possible to differentiate between bacterial and viral acute respiratory infections based on clinical signs or radiology (www.who.int). The inability to identify a potential pathogen is therefore a significant problem in the hospitals as clinical management of the patient is difficult without a proper diagnosis. Thus, it is extremely important to improve the techniques used for the identification of pathogens to detect both known and unknown causative agents.

1.2.2 Epidemiology of Viral Pneumonia

Many studies have shown viruses to outnumber bacteria in childhood community-acquired pneumonia, especially in children < 2 years of age. The most frequently isolated viruses are respiratory syncytial virus (RSV), influenza A and B, parainfluenza type 1, 2, and 3 and adenovirus (Kafetzis, 2004: Farha *et al.,* 2005; Mahony, 2008). Enteroviruses and human rhinovirus (HRV) are occasionally found. Contribution of human metapneumovirus (hMPV) to viral pneumonia is still fairly uncertain but a study conducted in 2004 (Williams *et al.,* 2004) identified hMPV to be in 20% of previously diagnosed virus-negative lower respiratory tract illness in children in the USA, and 8% of the cases where it was found, had a clinical diagnosis of pneumonia. Two "new" viral infections that are creating concerns are the severe acute respiratory syndrome (SARS) and influenza A (Burgner *et al.,* 2005). SARS, a newly described disease caused by infection with a coronavirus with a high mortality rate was reported in several continents in the year 2003 (Liu *et al.,* 2005; Muller *et al.,* 2006; Porten *et al.,* 2006). Since then there have been constant fears that it could make a comeback in the near future as a potential cause of significant paediatric infections.

On a more recent note, there are mounting concerns that avian influenza, (Influenza A H5N1) a viral infection contracted from infected birds and poultry, which causes significant morbidity and mortality, will spread and

cause an epidemic and possibly also a pandemic (Burgner *et al.,* 2005). Therefore, it is extremely important to undertake surveillance measures and to track the worldwide spread of these diseases to try and put a stop to these infections. In addition, surveillance to correlate and track the progress may also lead to a global effort in combating the microbes as in the case of the avian bird flu. Furthermore, these epidemiological studies can also pick up any mutations and raise the awareness of these newly evolved strains.

A recent London study indicated that influenza A caused approximately one-third of viral community-acquired pneumonia and 16% of all community-acquired pneumonia. Influenza B on the other hand tends to affect those with underlying medical problems (Burgner *et al.,* 2005). Influenza is highly contagious and undergoes rapid antigenic change which causes annual or near-annual seasonal epidemics of febrile respiratory disease affecting all age groups. In addition, influenza A viruses frequently initiate explosive global epidemics of disease known as pandemics. Since 1977, influenza A (H1N1), influenza A (H3N2) and influenza B viruses have been in worldwide circulation. Since early 2004, influenza A (H5N1) virus, which caused 6 deaths in Hong Kong in 1997 is making a comeback in Vietnam, Thailand, Indonesia and China among people in close contact with birds. There is now a great cause of concern as cases are now being reported in places like Japan, Russia, Turkey and Europe (Whitney *et al.*, 2004; Ng *et al.*, 2005).

A most recent threat is the emergence of "swine flu" which was later identified as a novel influenza A virus (H1N1). Since its first appearance in Mexico in March and early April 2009, it has caused significant morbidity and mortality in almost all regions including Africa, the Americas, the Eastern Mediterranean, Europe, South-East Asia as well as the Western Pacific accounting for up to over 182,166 cases and 1799 deaths as of 13 August 2009 (www.who.int). The efficient and rapid spread of this infection was immediately noted by the medical profession and though the illness associated was later noted to be generally self-limiting and uncomplicated; a substantial number of cases of severe disease and death have been reported in previously healthy young adults and children (MMWR, 2009). With experiences gathered from the SARS outbreak in 2003, concerted responses by health organizations throughout the world including World Health Organization (WHO) and Centers for Disease Control and Prevention (CDC) in Atlanta, United States to monitor and compile surveillance data were quickly put in place. The key concerns include assessment of the potential impact on public health; clinical progression of disease, including rate and types of complications for different age and risk groups; as well as information on virus transmissibility. Assessment of potential disease severity associated with this novel virus is important as the information helps to shape decisions on prevention strategies that slow the spread of the infection until vaccines are available as a form of prophylaxis (Fiore *et al.*, 2009).

1.2.2.1 Respiratory Syncytial Virus (RSV)

1.2.2.1.1 The Virus

RSV was first discovered in 1956 by Morris and colleagues while working with chimpanzees. They named the agent causing mild upper respiratory tract illness “chimpanzee coryza agent” as human contacts working with chimpanzee were infected by them. In 1957, Chanock and colleagues upon studying cytopathic effects of the agent in tissue culture renamed it as “respiratory syncytial virus” (Ogra, 2004).

RSV is a pleomorphic, enveloped, cytoplasmic virus containing single-stranded, negative sense RNA. The RNA is associated with viral proteins, consisting of a nucleocapsid core that is packaged within a liquid envelope. RSV genome is approximately 15.2 kb and is classified in the genus *Pneumovirus,* which belongs to the family *Paramyxoviridae*. (Figure 1.1). It displays minimal antigenic heterogeneity with only two major groups A and B identified. The antigenic differences for these two groups are in the glycoprotein (G, attachment protein), disulfide-bonded glycoprotein (F, fusion protein), nucleoprotein (N), and phosphoprotein (P). The G protein is the most variable protein with only 53% homology in the amino-acid sequences between the proteins of A and B groups. In contrast, the F and N proteins

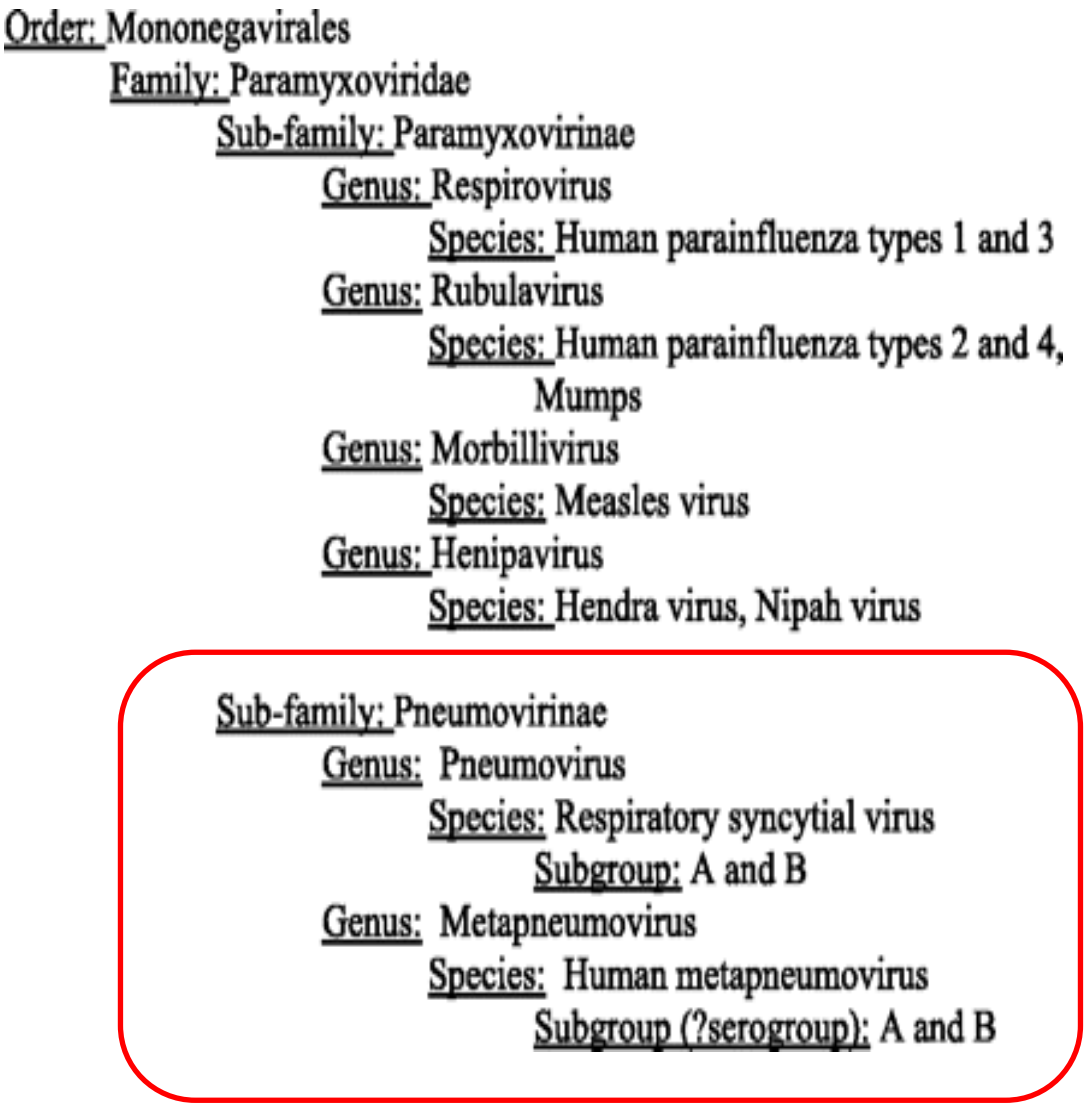


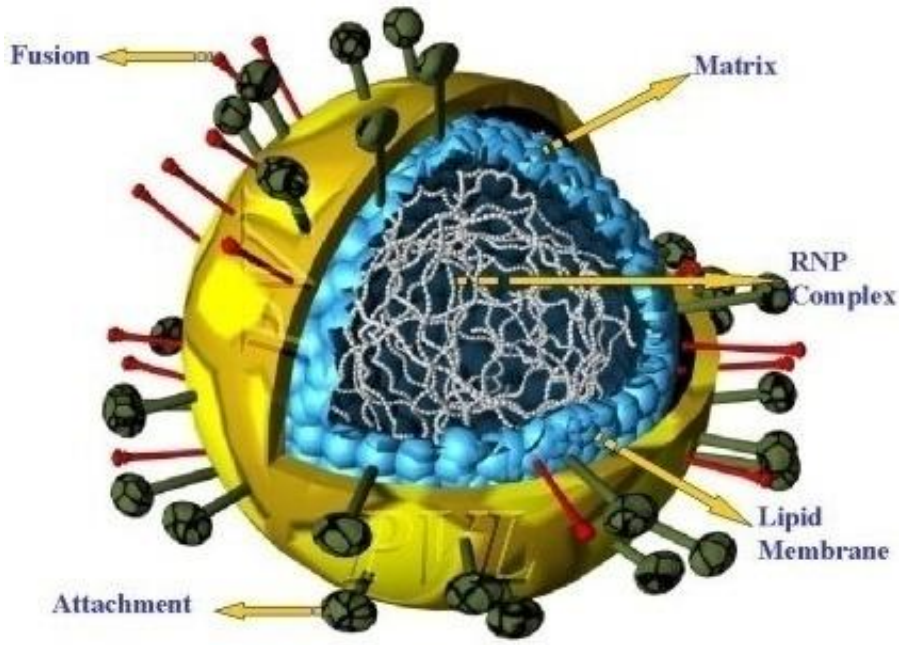
Figure. 1.1. Human pathogens in the sub-family *Pneumovirinae*.
Genuses included in the *Pneumovirinae* sub-family are shown in the red box. (Adapted from Kahn, 2006)

have a high degree of genetic and antigenic homology (Ogra, 2004) (Figure 1.2).

Currently, not much emphasis has been given to differentiate RSV type A from RSV type B in the clinical setting as the current immunological method used in the routine microbiology laboratory is unable to do so (Ahluwalia *et al.,* 1988; Thomas *et al.,* 1991; Henrickson *et al.,* 2007). Furthermore, there are still limited data to suggest that treatment for both RSV types should differ (Ogra, 2004) though there is indication that infections caused by RSV type A subgroup tend to be more severe than RSV B (Perkins *et al.,* 2005; Fodha *et al.,* 2007). Epidemiological studies have classified RSV A into eight genotypes and group B into four genotypes (Kuroiwa *et al.,* 2005; Zlateva *et al.,* 2007; Reiche *et al.,* 2009), though one study conducted in Japan identified new mutants of RSV B in Sapporo, which suggested that RSV strains may undergo geographical and temporal clustering while participating in RSV genetic evolution in a global setting (Kuroiwa *et al.,* 2005).

RSV displays minimal antigenic heterogeneity, although, two antigenically distinct subgroups, group A and B, are known. Epidemiologic studies have shown that there are three types of RSV epidemics, those in which group A or

(a)



(b)

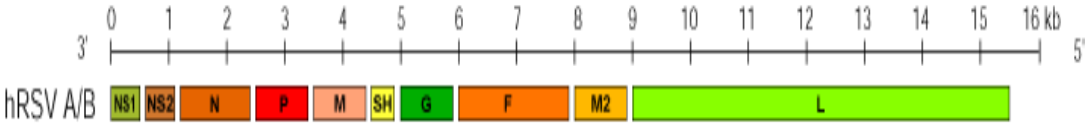


Figure. 1.2. (a) Illustration of the respiratory syncytial virus. Diagram shows fusion (F) protein and attachment (G) protein. (Adapted from http://medgenmed.medscape.com/viewarticle/431615_print)
(b) Schematic representation of the respiratory syncytial virus genome. (Adapted from http://www.uq.edu.au/vdu/VDUHumanRespiratory SyncytialVirus.htm)

group B viruses were dominant and those in which both groups circulate concurrently. In children, subgroup A strains were detected at least three times as often as subgroup B in most years. Moreover it has been shown that the course of disease for infections with RSV strain A is usually more severe (Borg *et al.,* 2003; Fodha *et al.,* 2007).

The major differences between subgroup A and B are on the glycoprotein (G), fusion protein (F), nucleoprotein (N) and phosphoprotein (P). Both F and G proteins have several distinct antigenic sites. Recent data have shown that G protein sequences may differ by 20% in different group A lineages and 9% in different group B lineages (Ogra, 2004).

1.2.2.1.2 The Disease, Treatment and Prevention

Among pathogens that cause pneumonia, viruses have been reported to cause up to 90% of pneumonia, especially in the first year of life and this percentage decreases to approximately 50% by school age (Lichenstein *et al.,* 2003; Farha *et al.,* 2005) and the virus that most frequently causes pneumonia in children requiring hospitalization is RSV. Infection of previously healthy children with this virus usually results in mild upper respiratory tract disease in these children that resolves spontaneously, but in a minority of cases (usually infants < 3 months) and high-risk paediatric groups (preterm infants,

children with pulmonary or cardiac disease and immunocompromised children), severe lower respiratory tract infection with considerable illness and death is a more frequent occurrence (Simoes, 2002). Potential long-term consequences of LRTI caused by RSV include possible associations with (and future development of) asthma, allergies, and other chronic pulmonary illness including recurrent wheezing (Checchia, 2008; Stensballe *et al.*, 2008).

In a review paper presented by Sinaniotis (2004), the incidence for this viral infection ranges from 24% to 78% in children with pneumonia. Re-infection with RSV is common in older children, adults and in the elderly because unlike a number of other infectious diseases, infection with RSV does not confer protective and enduring immunity (Simoes, 2002; DeVincenzo, 2007; www.CDC.gov). Research for effective treatment strategies for RSV has been ongoing for almost four decades with few successes (Simoes, 2002; Kafetzis, 2004; Checchia, 2008). Tests of the first developed vaccine on human children ended with disastrous results when natural RSV infection developed in those children (up to 80% of the children were hospitalized, and two children died) (Mohapatra *et al.*, 2008). Since then, development of an effective RSV vaccine has met with many challenges including the need to induce immunity to the multiple strains of RSV (Simoes, 2002). Also, because the children at highest risk are those younger than 3 months, vaccination would ideally take place when the child is still a newborn (≤ 1 month old), raising concerns regarding possible interference by maternal antibodies to live vaccines. A further concern is that since natural infection does not prevent

reinfection, a series of boosters may be needed for protection against subsequent disease (Simoes, 2002; Mohapatra *et al.*, 2008). A recent study by Schickli *et al.*, (2009) defined a vaccine candidate for RSV-naive infants as one that must provide immunogenicity in the presence of maternally acquired antibodies, avoiding enhanced disease and have minimal reactogenicity. As live RSV infection does not potentiate for enhanced disease and elicits systemic and mucosal immune response, live attenuated RSV vaccine candidates are currently preferred (Schickli *et al.*, 2009).

Currently, very little is available in terms of specific therapy for RSV infection, although, fluid resuscitation and supplemental oxygen in infants are fortunately effective in the management of these infections. Even in cases of respiratory failure, mortality is uncommon if assisted ventilation is provided early on in the course of illness (Welliver, 2004). In a recent study conducted by Wang *et al.*, (2008), prophylaxis treatment with palivizumab, a humanised monoclonal antibody against RSV F protein has shown to be clinically effective for reducing the risk of serious lower respiratory tract infection caused by RSV infection and requiring hospitalization in high-risk children. However, the high cost of palivizumab coupled with epidemiologic evidence of the link between RSV and reactive airway disease limit the use of this prophylaxis as a treatment against severe RSV infection (Mohapatra *et al.*, 2008).

1.2.2.1.3 Epidemiology and Seasonality

In temperate regions of the world, there is a clear seasonal variation in the occurrence of RSV infections during the cold winter months. The reasons that have been suggested are crowding of susceptible individuals indoors in winter and the cooling of the nasal passages with concomitant decrease in respiratory defence. However, in the tropics, this association is less obvious as there is generally less fluctuation in ambient temperature. A 5-year review of 9635 paediatric patients conducted in Hong Kong displayed a clear seasonal pattern where an increase of cases of RSV infections coincided with an increase of rainfall, humidity and temperature (Chan *et al.,* 1999; Shek *et al.,* 2003) during the April to September months. In another retrospective study carried out in Singapore from 1990 to 1994 observed peaks occurring from March to August where temperatures were higher but relative humidity and rainfall was lower (Chew *et al.,* 1998; Shek *et al.,* 2003). A more recent study reported Taiwan seasonality of RSV showing a biennial pattern with peaks occurring in the spring and fall (Lee *et al.,* 2007). In general, although the average temperature of the tropics is higher and the seasonal change in temperature is less than in temperate regions, these regions do experience local variations in temperature, humidity and rainfall. Through the many studies conducted, it is clear that RSV infection is seasonal in the tropics and the weight of evidence points to the fact that it is more common during the rainy season. During the rainy season, children tend to be kept indoors, and the resultant crowding may

account for the increase in incidence of RSV during this period. Another possibility is that high humidity may be conducive to viral survival by preventing drying and loss of infectivity of the virus. RSV is also known to be a labile virus, and does not survive well at high temperature, which may explain the relationship with cooler months (Shek *et al.*, 2003; Welliver *et al.*, 2007). However, the timing and severity of RSV circulation in a given community can vary from year to year (www.CDC.gov).

1.2.2.2 Human Metapneumovirus (hMPV)

1.2.2.2.1 The Virus

hMPV, a respiratory tract pathogen discovered in Denmark in 2001, causing clinical symptoms resembling RSV infections in the upper and lower tract disease, has been associated with serious illness in the young, among the immunosuppressed and in the chronically ill (Alto, 2004; Kahn, 2006; Arabpour *et al.*, 2008). Since then, hMPV has been reported worldwide and samples dated as far back as 1958 were retested and found to be positive for this virus. It is believed that initial infection occurs during early childhood, and repeated infections throughout life impart only transient immunity. Co-infection with other respiratory viruses occurs, and simultaneous infection

with hMPV and RSV has been postulated to cause severe disease. The disease is believed to be more serious in infants and in those with chronic illnesses and primary infection with the virus is believed to cause the most severe symptoms (Greensill *et al.*, 2003; Semple *et al.*, 2005; Kahn, 2006). There are at least two different genetic lineages of hMPV and viral genome heterogeneity may also allow for incomplete immunity and repeated infections (Alto, 2004; Kahn, 2006). It is difficult to be certain of the contribution of hMPV, but it has been identified at between 1.5 and 43% and up to 56.4% in children suffering from ARTIs (Arabpour *et al.*, 2008).

hMPV virus is a member of the genus *Metapneumovirus*, a branch of the family *Paramyxoviridae*, and is genetically similar to, though distinct from, the avian pneumovirus (APV). The family *Pneumovirinae*, which includes both the genus *Pneumovirus* and the genus *Metapneumovirus*. Although APV and hMPV are related to the human and animal RSV viruses, they differ in that the gene order in the nonsegmented genome is slightly altered and the APV/hMPV are lacking the 2 non-structural proteins NS1 and NS2 located at the 3'end of RSV genomes. The hMPV genome is predicted to encode nine proteins in the order 3'-N-P-M-F-M2-SH-G-L-5' (the M2 gene is predicted to encode 2 proteins, M2-1 and M2-2, using overlapping open reading frames, as in RSV). The genome also contains noncoding 3' leader, 5' trailer and intergenic regions, consistent with the organization of RSV (Figure 1.3) (Crowe, 2004; Kahn, 2006).

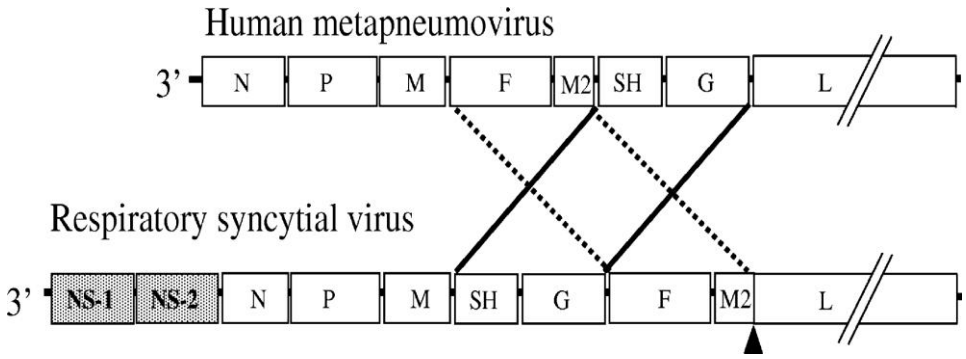


Figure. 1.3. Genomic maps of the *Pneumovirinae*. Genomic maps of the negative-sense, single-stranded RNA genomes of hMPV and RSV are displayed in the 3'-to-5' orientation. In hMPV, the F and M2 genes are 3' to the SH and G genes, whereas in RSV, the order of these genes is reversed. The RSV genome encodes two nonstructural proteins, NS-1 and NS-2 (shaded), that are not present in the hMPV genome. The M2 genes of both viruses carry two ORFs (M2-1 and M2-2) (not shown). The M2 and L genes overlap in the RSV genome (triangle). The L gene of each virus, encoding the viral RNA-dependent RNA polymerase, comprises two-thirds of the viral genome and is shortened for figure clarity. The genomes are not drawn to scale (Adapted from Kahn, 2006).

Based on several studies from different world regions, phylogenetic analysis of the fusion protein (F) gene from hMPV showed the presence of only two genetic clusters or groups (known as cluster A and B), suggesting that a relatively homogenous population of hMPV is circulating throughout the world (Ebihara *et al.,* 2004; Peret *et al.,* 2002; van den Hoogen *et al.*, 2002). However, recently, the hMPV glycoprotein (G) gene associated with the attachment of the virus, was shown to be highly variable, particularly in the extracellular domain, as a result of nucleotide substitutions, insertions, and the use of alternative termination transcription codons. Data obtained showed levels of amino acid identity ranging from 31 to 37% between the G proteins of the two hMPV clusters and the presence of additional diversity within clusters (Ludewick *et al.,* 2005; Bastien *et al.*, 2004). As with RSV, the G protein which is involved in neutralizing and protective immunity and the high percentage of nucleotide changes that resulted in amino acid changes suggests that there may be a selective advantage to G protein changes, which may help the virus to evade the immune system (Bastien *et al.,* 2004).

1.2.2.2.2 The Disease, Treatment and Prevention

hMPV is capable of infecting people of all age groups. It generally causes a influenza-like illness with increased morbidity and mortality in young children, infants, the elderly and the immunocompromised patients (Sloots *et*

al., 2008). Evidence from many studies has demonstrated that hMPV is responsible for a substantial proportion of lower respiratory tract infections in infants and young children and is second only to RSV as a cause of brochiolitis in early childhood. The incidence of hMPV-associated lower respiratory tract infections (LRTIs) in young children varies with geographical location and time of year and incidence is estimated to range from 5 to 15% in most studies (Kahn, 2006). The clinical manifestations of hMPV infection in young children are indistinguishable from the clinical manifestations of RSV infection. Features of hMPV infection included tachypnea, fever, cough, hypoxia, and changes on chest radiography. Asymptomatic infection with hMPV in infants and young children appeared to be uncommon with approximately 10% of these children hospitalized with LRTI. The most common diagnoses are bronchiolitis, pneumonia, and bronchitis. hMPV has also been associated with acute asthma exacerbations and wheezing is a common symptom observed in children with hMPV-associated LRTI (Chano *et al.,* 2005; Kahn, 2006; Klein *et al.*, 2006; Ordas *et al.,* 2006).

The overall rates of hMPV disease in adults are likely lower than those observed in children. However, hMPV has been associated with influenza like illness, bronchitis, pneumonia, and exacerbations of both asthma and chronic obstructive pulmonary disease (COPD) in adults. The major risk factor for hMPV-associated respiratory tract disease in adults without COPD is

advanced age and underlying cardiopulmonary disease. Dyspnoea was also more likely in elderly adults than in young children infected with hMPV (Kahn, 2006).

As with other respiratory viruses, antiviral drug therapy is difficult to implement effectively in actual practice, because viral shedding is usually already decreasing at the point in the course of infection when patients present to their medical providers. Nevertheless, ribavirin and intravenous immunoglobulin, which have activity against RSV, were tested against hMPV *in vitro* and found to have equivalent antiviral activity against these viruses. In addition, heparin and the sulphated siayl lipid have also been shown to have activity against hMPV. In terms of vaccine development, several promising vaccine candidates have already been tested in animal models. A live recombinant human parainfluenza virus that contains the hMPV F gene has been shown to induce hMPV-specific antibodies and to protect experimental animals from hMPV challenge. A chimeric bovine/human parainfluenza virus 3 expressing the hMPV F elicits neutralizing antibodies against both parainfluenza virus and hMPV. However, the results of these animal challenge studies should be interpreted cautiously as there are many limitations of small-animal model testing. In the case of hMPV vaccines, the pathogen is highly host restricted (Crowe, 2004; Kahn, 2006).

1.2.2.2.3 Epidemiology and Seasonality

Since its discovery in 2001, hMPV has been identified in every continent with an almost worldwide distribution (Kahn, 2006; Sloots *et al.,* 2006; Loo *et al.,* 2007, Ong *et al.,* 2007). In countries with moderate climate temperate, hMPV has a seasonal distribution overlapping RSV circulation. Most of the cases reported are during winter and early spring (Ljubin-Sternak *et al.,* 2008). In many other communities, hMPV has been detected throughout the year, albeit at lower levels during the late spring, summer, and fall (Kahn, 2006). Genetic studies on hMPV have demonstrated the presence of 2 distinct hMPV groups (A and B) and subgroups (A1, A2, B1 and B2) within these groups; with recent evidence showing the existence of multiple lineages (Ludewick *et al.,* 2005). The major differences between A and B genotypes are nucleotide polymorphisms, and the G and H proteins contain the highest concentration of these polymorphisms. Strain-to-strain variations occur mostly in the G gene which results in significant amino acid variability, insertions that retain the reading frame and the use of alternate transcriptional termination codons. Overall, there is 32 to 27% amino acid identity of the G protein between A and B genotypes of hMPV (Bastien *et al.,* 2004).

Phylogenetic analysis of strains of hMPV reveals that the epidemiology of hMPV is complex and dynamic. Strains of hMPV differ from community to community and strains identified in other locations in different years. One

example is that the prototype identified in The Netherlands that is genetically similar to strains identified in Australia, New Haven and Canada in different years (Kahn, 2006). In any given year, viruses of both genotypes (and both subgroups in each genotype) can circulate or co-circulate with predominant strains varying from location to location and from year to year. For example, in St. Louis, Missouri, the predominant genotype of hMPV switched in consecutive years from genotype A to genotype B (Agapov *et al.*, 2006).

In 2004, a variant of hMPV, isolated from a 6 1/2 –year-old girl with an acute exacerbation of asthma, was found to be genetically distinct from viruses of the four lineages of hMPV when analysing or comparing the N gene sequences (Kahn, 2006). In 2009, a novel A3 sub lineage was proposed by Escobar *et al.,* (2009), where most Chilean strains isolated were found to be in this new cluster.

Infection with hMPV appears to be common in childhood. By the age of 5 years, more than 90% of individuals screened have evidence of hMPV infection. The seroprevalence of hMPV-specific antibody in adults is nearly 100% (van de Hoogan *et al.,* 2001; Kahn 2006). The seroprevalence of hMPV-specific antibody in infants <3 months of age is >90%, indicating that maternally derived antibodies are present in young children. Whether this hMPV-specific antibody protects against infection or lessens the severity of illness remains to be determined.

1.2.2.3 Human Rhinoviruses (HRVs)

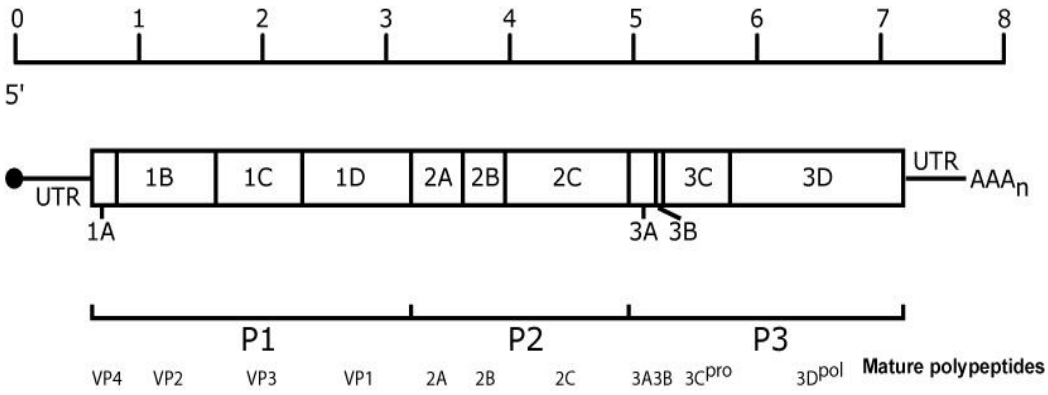
1.2.2.3.1 The Virus

HRVs are classified in the family *Picornaviridae* with currently more than 100 serotypes recognized by the Collaborating Rhinovirus Laboratories (Patick, 2006; Dreschers *et al.*, 2007; Arden *et al*., 2009). It was thought that there were only two different species of HRV: HRV A and HRV B circulating in the community, however, with the use of molecular techniques, many divergent and previously uncharacterized HRV strains were identified, including HRV C (McErlean *et al.*, 2007; McErlean *et al.*, 2008). The reason for this diversity was thought to be determined by an altered ultrastructure of viral capsids (Dreschers *et al*., 2007). The capsids is composed of four proteins (viral proteins (VP), VP1-VP4), which arise from a single polyprotein generated from the viral mRNA genome functioning as an mRNA (Dreschers *et al*., 2007; Mackay *et al*., 2008).

HRVs are single positive-stranded RNA viruses of approximately 7200 base pairs (bp) and tend to be adenine and uracil rich. They belong to the family *Picornaviridae* and are closely related to human enteroviruses (HEVs), another genus of the same family. The genome organization of *Picornaviridae* is conserved among the family with a long 5'-untranslated region (UTR), a

single open reading frame (ORF) encoding a polyprotein, a short 3' UTR, and a poly(A) tail. The UTRs are known to perform regulatory functions that permit genome duplication and production of a single, multi-domain, proteolytically processed 'polypeptide". The mature capsid proteins VP1 (34-36 kDa), VP2 (27-30 kDa) and VP3 (24-28 kDa) all exist as a convulated set of protein sheets and loops (Figure 1.4) (Stirk and Thornton, 1994; Ledford *et al*., 2005). The loops protrude beyond the external capsid surface and contain important, often discontinuous, antigenic sites. Four neutralizing antibody immunogenic (NIm) regions have been identified on the HRV-14 and HRV-16 virion; NIm-1A (located in VP1), NIm-1B (VP1), NIm-II (VP2 and VP1) and NImIII (VP3 and VP1). Antigenic sites (A, B and C) have also been identified on HRV-2, a minor group virus (Dreschers *et al.*, 2007; Mackay, 2008). It was noted that VP1, VP2 and VP3 were exposed structural proteins while VP4 was an internalized structural protein of approximately 7kDa situated within a 2.5 nm depression along the fivefold icosahedric axis of the virion, often referred to as "the canyon". The genomic region encoding the peptide residues that form this structure is more conserved than regions encoding any other structure on the virion surface (Rossmann *et al.*, 2002; Dreschers *et al.*, 2007; Mackay, 2008).

(a)



(b)

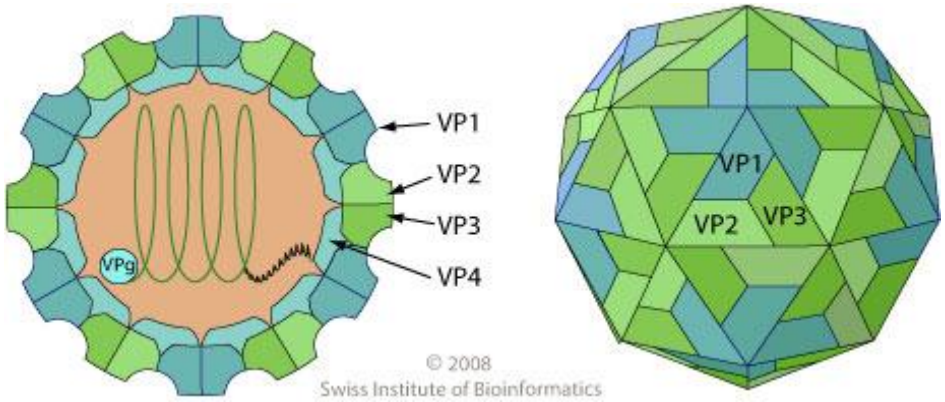


Figure.1.4. (a) Schematic representation of the human rhinovirus genome. Based on GenBank accession no. NC_001490 for HRV-QPM. (Adapted from www.uq.edu.au/vdu/VDURhinovirus.htm) **(b) Illustration of the human rhinovirus.** Rhinoviruses are composed of a capsid, that contains four viral proteins VP1, VP2, VP3 and VP4.[1][2] VP1, VP2, and VP3 form the major part of the protein capsid. The much smaller VP4 protein has a more extended structure and lies at interface between the capsid and the RNA genome. There are 60 copies of each of these proteins assembled as an icosahedron. Antibodies are a major defense against infection with the epitopes lying on the exterior regions of VP1-VP3. (Adapted from www.expasy.ch/viralzone/all_by_species/33.html)

Picornaviruses are recognised by a variety of receptors. The capsid of the majority of HRVs (89 "major" group) interacts with the amino-terminal domain of the 90 kDa intercellular adhesion molecule (ICAM-I; CD54). Receptor binding destabilizes the HRV capsid, probably by dislodging the "pocket factor" and initiating uncoating. The minor group of viruses employ members of the low density lipoprotein receptor (LDLR) family to attach to cells. Binding of the VLDL-R occurs outside of the canyon's Northern face (site between VP1 and VP3) employing a different destabilization mechanism for virus coating (Dreschers *et al.,* 2007; Mackay, 2008).

1.2.2.3.2 The Disease, Treatment and Prevention

HRVs are a group of pathogens almost all individuals are likely to encounter at least once by the age of 2 (Savolainen *et al.,* 2003). They are also the most frequent viruses associated with respiratory infections in humans and have a global distribution (Pitkaranta *et al.,* 1998; Tapparel *et al.,* 2007). The major presenting illness of HRV infection is the common cold syndrome, with approximately one-third of infections either minimally symptomatic or sub-clinical. Symptoms of the infection usually appear 1-4 days after incubation and peak 3-7 days after onset of infection. The infection is self-limiting and patients usually make a full recovery within a 2 week period (Nikolaos *et al.,* 2000; Turner, 2007). However, it is now becoming increasingly clear that the

relatively benign nature of HRVs is only partly true. Emerging data are associating them with more severe diseases, such as acute otitis media in children and sinusitis in adults (Greenberg 2003; Papadopoulos, 2004; Turner, 2007). There are also suggestions that it can also infect the lower respiratory tract and therefore account for lower respiratory tract symptoms such as pneumonia, wheezing in children and exacerbations of asthma and chronic obstructive pulmonary disease (COPD) in adults (Rawlinson *et al.,* 2003; Miller *et al.,* 2007; Turner 2007).

Until recently, isolation of HRVs by the viral culture method has been the only diagnostic method available (Korppi *et al.,* 2004). It is only through the use of polymerase chain reaction (PCR) methods developed for HRVs introduced lately that the overall disease burden associated with this infection emerged. In a study by Arden *et al.* (2006), HRV was detected by molecular methods in 44.4% of people with suspected acute lower respiratory tract infection while another study by Jartti *et al.* (2004) placed HRV infective rate at 24% for children with acute expiratory wheezing. It is now widely accepted that rhinovirus is an important cause of asthma exacerbations in school-age children and may account for up to 70% of all cases in this age group. High prevalence of rhinovirus in hospitalized children and adults has also been documented recently and Peltola *et al.* (2008) detected 46 (28%) of 163 hospitalizations in these children. The major contributing factors of hospitalizations of children wheezing are due to rhinovirus. In a recent study

by Hamano-Hasegawa *et al.,* (2008), 1700 pediatric patients with community-acquired pneumonia were analysed and rhinoviruses were the leading viral cause of this infection accounting for 14.5% of all pneumonia cases.

Transmission of HRV to susceptible individuals is primarily by direct contact but virus can also be spread by small or large particle aerosols (Patick, 2006; Turner, 2007; Mackay, 2008). HRVs have a relatively low optimum in vitro temperature for growth, 33°C, considered to reflect an evolutionary adaptation to the environment of the nasopharyngeal region, though some HRVs can also multiply at higher temperatures (Savolainen *et al.,* 2003; Turner, 2007). The virus is deposited on the nasal or conjunctival mucosa and is transported to the posterior nasopharynx by ciliated epithelial cells. In the nasopharynx, the virus attaches to epithelial cells via either the intracellular adhension molecule-1 (ICMA-1 for HRV major serotypes) or low-density lipoprotein (LDL-1 for HRV minor serotypes) receptors and initiates infection (Mackay, 2008).

Chemotherapeutic approaches toward HRV-induced illnesses have been the focus of intense research spanning several decades. Comprehensive reviews on small molecule inhibitors that target specific virus functions including virus attachment, uncoating, virus RNA replication and viral protein synthesis and processing have been extensively detailed. However, despite extensive research efforts that have led to the discovery of many potent antiviral agents,

no drug today has been approved for the treatment of HRV-induced illness (Turner 2005; Patick 2006; Mackay, 2008). In the absence of specific therapies, treatment relies on symptomatic remedies directed at specific symptoms. In recent years, alternative treatments for common cold have attracted significant interests. One potential compound is zinc as it was observed to be an inhibitor of 3C protease, an enzyme essential for rhinovirus replication was documented (Turner, 2001). Since there are no pharmacologic or vaccine interventions for prevention of rhinovirus infections, recommendations for prevention of these infections are limited to efforts to prevent transmission of the virus from infected susceptible individuals. As noted, direct hand-to hand contact appears to be the most common and efficient mode of rhinovirus transmissions. This observation led to the assumption that simple handwashing might have a beneficial effect on the incidence of these infections, and ethanol decontamination is shown to be more effective in removing rhinovirus than water from the hands (Sattar *et al.*, 2000; Turner, 2005).

1.2.2.3.3 Epidemiology and Seasonality

With more than 100 serotypes, HRVs are the most common cause of acute respiratory illnesses in humans and have a global distribution. These infections begin in early childhood and continue throughout the adult years.

The rates are highest in young children; with more than 90% of children experiencing at least one HRV infection by 2 years of age (Savolein *et al.*, 2003). The rate is also increased in young women, presumably because of virus spread from children to their mothers and decline after middle age. Rhinovirus infections occur all year, but there are seasonal peaks of incidence usually September to November, and again in the spring, from March to May. During these periods of increased incidence, up to 80% of common cold illness may be associated with a documented rhinovirus infection (Monto, 2002; Papadopoulos, 2004; Turner, 2005).

HRVs immunologically distinct serotypes have been shown to correlate with VP1 gene sequences and classification of HRVs are according to several parameters, including receptor specificity, antiviral susceptibility, and nucleotide sequence homologies. For the latter, phylogenetic analysis of VP4/VP2 sequences has shown that all but one HRV serotype belonged to two different species, HRV-A (comprising 74 serotypes) and HRV-B (compromising of 25 serotypes). The same topologies were also observed upon the subsequent analysis of VP1, partial 2A, and 3D-coding regions. The only exception was HRV 87, which was found to belong to human enterovirus D (HEV-D) species that has both rhinovirus and enterovirus features (Blomqvist *et al.*, 2002; Lau *et al.*, 2007; Mackay, 2008). In a recent study by Tapparel *et al.*, (2007), phylogenetic analysis of 12 HRV-A and HRV-B complete genomes suggested that HRV-B and HEV diverged from their

common ancestor after their separation from HRV-A. During the winter of 2004, a new rhinovirus genotype was identified in respiratory samples of patients from New York with influenza-like illness, while in 2007, McErlean *et al.*, (2007) identified a novel HRV-QPM in infants with bronchiolitis from Queensland hospitals. Since then, complete genome analysis of these novel strains were found to belong to a newly identified HRV species, HRV-C which is not only circulating worldwide, but was also found to be an important cause of febrile wheeze and asthmatic exacerbations in children requiring hospitalization (Lau *et al.*, 2007; McErlean *et al.*, 2007; McErlean *et al.*, 2008).

1.2.2.4 Epidemiology of RSV in Indonesia and Southeast Asia

Southeast (SE) Asia is a sub-region of Asia and geographically, is situated at the south of China, east of India and north of Australia and are divided into two sub-regions, namely Mainland SE Asia (or Indochina) and Maritime SE Asia (or the similarly defined Malay Archipelago). Except for Brunei and Singapore, which are considered to be developed countries, the rest of the ten SE Asia countries (Cambodia, Indonesia, Laos, Malaysia, Myanmar, Papua New Guinea, Philippines, Thailand, Timor-Leste and Vietnam) are classified as emerging and developing countries by the International Monetary Fund in

their 2009 World Economic Outlook Report (http://www.imf.org/external/pubs/ft/weo/2009/02/weodata/groups.htm).

Though many studies had identified ALRTIs as an important cause of morbidity in infants and children in developing countries (Singh, 2005; Omer *et al.*, 2008; Brooks *et al.*, 2010), there are few reports on RSV lower respiratory illness disease burden existing from rural areas of the developing countries, including Indonesia. In the few studies conducted in Indonesia, Djelantik *et al.,* (2003) reported the incidence of RSV-positive lower respiratory illness with hospitalization in Lombok, Indonesia estimated at 23%, while Omer *et al.,* (2008) reported an RSV infection rate f 20.1% when testing 3688 episodes of lower respiratory illness in hospitalized patients. In another denominator-based surveillance study conducted in Bandung, Indonesia, the incidence of RSV determined at home visits was cited as 23% for children under 1 year of age (Robertson *et al*., 2004). Though the prevalence rate of RSV in these three Indonesian studies were somewhat comparable to the 16% in the United States, the proportion of severe LRIs was higher in Indonesia (<45% among children aged <5 years and 64% among children aged < 1 year) with death occurring frequently (Djelantik *et al*., 2003; Robertson *et al.,* 2004). Currently, RSV has been reported in many SE Asia countries including Malaysia, Philippines, Singapore, Thailand and Vietnam (Capeding *et al*., 1994; Chan *et al*., 2002; Suwanjutha *et al*., 2002; Loo *et al*., 2007; Yoshida *et al*., 2010).

1.2.2.5 Emerging Respiratory Pathogens

Recent advances in molecular biology have greatly improved the detection of viral respiratory pathogens. Yet, even with the most sensitive techniques, only 40-60% of infections were able to be associated consistently with a causative microorganism (Louie *et al*., 2005), suggesting that additional respiratory pathogens are likely to exist (van den Hoogen *et al*., 2001). Since 2001 when van den Hoogen *et al.,* (2001) described the presence of hMPV, other previously undescribed viruses have been identified by analysis of clinical specimens from human respiratory tract using novel molecular methods such as VIDISCA, pan-viral DNA microarrays and high throughput sequencing (Sloots *et al.,* 2008). Emerging respiratory pathogens detected in recent years using these new methods included three new human coronaviruses (HCoV); the severe acute respiratory syndrome (SARS) associated coronavirus in 2003 (Ksiazek *et al*., 2003), coronavirus NL63 in 2004; coronavirus HKU1 in 2005 (Woo *et al.*, 2005) as well as human bocavirus (HBoV) in 2005 (Allander *et al.,* 2005) and the recently described human polymaviruses KI (KIV) and WU (WUV) in 2007 (Allander *et al.*, 2007; Gaynor *et al.,* 2007).

Coronaviruses (CoV) infect many species of animals. Including humans and are classified into three distinct groups, with 229E and NL63 included in Group 1 coronaviruses and OC43 and HKU1 in Group 2. SARS-CoV represents an early split from Group 2 coronaviruses and is believed to have

originated from wild animals (Sloots *et al.*, 2008). First reports of an outbreak of a new respiratory illness involving "atypical pneumonia" were published in 2003 (Center for Disease Control and Prevention (CDC), 2003). Sequencing of the viral isolated showed the presence of a new human coronavirus and it was demonstrated that this virus was the etiologic agent for SARS by transmission of respiratory disease to nonhuman primates (Sloots *et al.*, 2008). The SARS epidemic was halted by a highly effective global public health response co-ordinated by the World Health Organization, and there is no evidence that SARS-CoV is currently circulating in humans. HCoV-NL63 on the other hand was detected in 2004 in a child with bronchiolitis in The Netherlands (van der Hoek *et al.*, 2004), whilst HCoV-HKU1 was detected in 2005 in an adult with chronic pulmonary disease in Hong Kong (Woo *et al*, 2005). After the initial discovery, many groups have reported the presence of these viruses in 1 to 10% of patients with acute respiratory tract infections, indicating a global presence (Sloots *et al.*, 2008). However, HKU1 and NL63 infections are generally not life threatening in healthy persons.

In the year 2005, HBoV was first described in Swedish children causing LRTI and since was reported in many countries in Europe, United States, Canada, Asia and Australia with prevalence rates ranging from 1.5% to 11.3% in these populations (Allander *et al*., 2005; Sloots *et al*., 2008). HBoV was most frequently detected in infants younger than 3 years of age and the presence of it in the upper respiratory tract was shown to be positively associated with

asthma, acute otitis media, and pneumonia (Arnold *et al*., 2006). Since the first initial description of KIV and WUV in respiratory specimens of children in Sweden, Australia and the United States, a number of studies have reported the presence of these viruses in a number of geographical locations, suggesting a global presence for these viruses (Allander *et al*., 2007; Gaynor *et al*., 2007, Sloots *et al*., 2008). One striking feature of early findings concerning KIV and WUV is their high rate of co-detection with other respiratory viruses. A co-detection rate of 74% has been observed for KIV (Bialasiewicz *et al*., 2008) and rates ranging from 68% to 79% for WUV (Han *et al*., 2007; Bialasiewicz *et al*., 2008). Although an etiologic role in childhood respiratory disease has been proposed for KIV and WUV (Allander *et al*., 2007; Gaynor *et al*., 2007), it is however difficult to assess the pathogenic role of these viruses and further studies will need to be completed before the role of KIV and WUV as respiratory pathogens can be confirmed (Sloots *et al*, 2008).

With the development of new molecular technology, our ability to detect and characterize new viral agents has greatly improved. However, for a significant proportion of clinical infectious disease of suspected viral origin, a pathogen still cannot be identified. These diseases remain technically challenging and prone to the amplification of non-viral related sequence artefacts. However, with continuing advances in molecular technology and the development of

more reliable, robust and reproducible molecular techniques, it seems certain that new potential viral pathogens of humans will be discovered.

1.2.3 Management of Paediatric Pneumonia

Measurement of morbidity in pneumonia may include: length of symptoms; time off school; for those admitted to hospital, time in hospital; duration of oxygen requirement; numbers requiring intensive care; time to recovery; as well as complications of the disease and of the treatment (Farha *et al.,* 2005). In general, suitable first-line antibiotics in children less than five years of age are penicillin- or cephalosporin-based and in those over five, macrolides may be considered. In severely ill children, good oxygenation is essential and oxygen may be delivered by mask, nasal prong or headbox (Russell-Taylor, 2000). In a study conducted in the United States (Michelow *et al.,* 2004), 154 children with pneumonia had an average of five days of symptoms prior to hospitalization. Forty percent of this cohort of patients received oral antibiotic therapy within the preceding 2-week period and 60% of these children received supplemental oxygen therapy for a median of 3 days. Ten children required assisted ventilation and there were 2 deaths. The median duration of hospitalization was 5 days and 4 children were readmitted within 30 days (Michelow *et al.,* 2004; Farha *et al.,* 2005).

One of the ways to control pneumonia is through the identification of the causative agent which will help to determine the most appropriate treatment for the children. However the difficulties in establishing a firm etiology for patients with pneumonia are well known. For bacterial pneumonia, culture of sputum is of uncertain value and samples of sputum are difficult to obtain from children; the degree of concordance between pharyngeal secretion cultures and lung aspirates cultures is poor; blood cultures yield a positive result in only 1% to 27% of patients with pneumonia, and isolation of bacteria is highly dependent on when the blood samples are collected, and which patients are recruited (Juven *et al.*, 2000; Nascimento-Carvalho, 2001). For optimal results, usually several techniques are used even for the identification of one pathogen. As yet, there are no sensitive and simple tests for the detection of the etiologic agents in biological fluids from patients with pneumonia (Nascimento-Carvalho, 2001) and this further emphasizes the need for better techniques to be developed to diagnose accurately the etiologic agents of infection.

The etiology of childhood community-acquired pneumonia is varied and establishing its etiology is complex. However, many studies have identified *S. pneumoniae* as the most frequently identified bacterial agent, followed closely by *H. influenzae*, RSV and *M. pneumoniae*, which all accounted for almost half of the pneumonia cases (Nascimento-Carvalho, 2001; Graham *et al.*, 2008). Besides *H. influenzae* type b, Non-type b *H. influenzae* is also an

important cause of pneumonia, as are numerous other serotypes of pneumococci (Nascimento-Carvalho, 2001). The immunogenicity of conjugate vaccines for *S. pneumoniae* and *H. influenzae* is type-specific. It is estimated that widespread use of currently available pneumococcal and *H. influenzae* type b (Hib) conjugate vaccines can prevent 30% of pediatric pneumonia. However, it is dependent on the serotype distribution of each of those pathogens in the region where vaccines are to be used. Therefore, in addition to detection of pathogens, epidemiological studies should also be performed to find out the predominant serotypes in a geographical region to give recommendation to the further development of vaccines and also to determine which strains the vaccines will be suitable for (www.who.int).

To summarise, viral respiratory infections are the most common diseases seen in humankind and affect mostly children. Viruses have been shown to cause up to 90% of pneumonia during the first year of life, and this percentage decreases to approximately 50% by school age (Kafetzis, 2004). RSV is the major viral respiratory pathogen that causes lower respiratory tract infection (bronchiolitis and pneumonia) in infants and young children worldwide. Other viruses commonly encountered include parainfluenza, influenza, adenovirus, coronavirus and other viruses have also been detected in children with respiratory infections. More recently, infections with a newly discovered virus, MPV have also been documented to cause RSV-like illnesses. In addition, human rhinoviruses, once thought to only result in the common cold has been

found to be involved in lower respiratory tract infections. In general, effective treatment and antiviral prophylaxis in either the active (vaccine) or passive (monoclonal antibodies) form for most of these viruses are not available. Palivizumab is the only approved agent to be used as prophylaxis against RSV infection. A positive treatment approach is good hydration and oxygen supplementation. While the best approach for prevention of viral spread and subsequent illness, is education of families and hospital staff in the importance of infection control (handwashing, avoiding passive smoke and crowds, hospital cohorting of infants suffering from lower respiratory tract infections, respiratory infection control measures) (Lichenstein *et al.*, 2003; Feldman, 2004; Kafetzis, 2004).

1.3 Techniques Involved In The Detection/Epidemiological Investigation Of The Agents Of Pneumonia

1.3.1 Introduction

Most children with mild to moderate pneumonia will be treated in the community. Criteria for hospital admission relate not only to severity assessment of the patient, but also to other factors including age, length of illness, facilities available both in the hospital and in the community. In general, children who have a toxic appearance for example, significant respiratory distress and an abnormal oxygen saturation need admission (Russell-Taylor, 2000; Graham *et al.,* 2008). Once a clinical diagnosis of pneumonia is made and a severely ill child is admitted to a hospital, several tests will be performed to diagnose and investigate the cause of pneumonia which is important in the management of the infection. Radiological assessment of the lungs of patients, full blood count, erythrocyte sedimentation rate, C-reactive protein as well as specific microbiological investigations may be carried out routinely (Coote *et al.,* 2000).

For epidemiological purposes it is important to attempt a microbiological diagnosis. However, based on the clinical symptoms, physicians usually ask for either a bacterial investigation or a viral one. In addition, identification of

an organism usually comes too late to dictate early treatment but the antimicrobial resistance pattern may guide subsequent antibiotic management of bacterial pneumonia (Coote *et al.,* 2000; Saiman, 2004).

1.3.2 Conventional Culture Techniques

Though taken as the gold standard, viral culture of nasopharyngeal samples collected from hospitalized patients suffering pneumonia to identify causative agents is laborious and time consuming and thus it is not routinely performed now unless in the case of the investigation of new and/or unknown viruses, for example in the detection of SARS (Curry *et al.,* 2006). Bacterial culture of specimens on the other hand is more commonly performed than viral culture, although even then it may not be very useful as correlation between colonization and infection is hard to establish at the time (Bannister *et al.,* 2000). Furthermore, some bacteria are either hard to culture or cannot be cultured by traditional culturing methods as in the case of mycoplasma and ureaplasma (Brooks *et al.,* 2004).

1.3.3 Microscopic Detection

In addition to microbiological culture methods, one other diagnostic method commonly performed for respiratory samples in routine clinical laboratory is microscopy. Traditionally, Gram stain done on a valid expectorated sputum specimen and observed through light microscope has served as a guide to the initial selection of antimicrobial therapy for patients with bacterial pneumonia (Anelavis *et al*., 2009). However, in a study by Ewig *et al.*, (2002), it was concluded that sputum Gram stain had a low diagnostic yield as a diagnostic tool in pneumonia. Nevertheless, there are a number of reasons why Gram stain testing is still widely accepted in hospital clinical laboratories, such as: (1) readily available, inexpensive and entails no risk to the patients, (2) does not require sophisticated equipment, (3) evaluation is completed within a few minutes and (4) many laboratories still think it is a valuable diagnostic method which provide prognostic information (Anelavis *et al*., 2009).

Viruses are too small to be viewed under the light microscopy, however, with the development of monocloncal antibodies (MAb) directed against specific viral proteins and linked to the fluorescent molecule e.g. fluorescein isothiocyanate (FITC), the ability to detect viruses by fluorescence microscopes are significantly enhanced (Kesson, 2007). Rapid immunofluorescent (IF) staining methods for the direct detection of viral antigens in respiratory specimens have excellent specificity and very good

sensitivity. Though, generally less sensitive than viral cultures, it however has a much faster turnaround time of 1-3 hours as compared to cultures which may take up to two weeks for recognizable cytopathic effects to take place (Landry *et al*., 2005; Kesson, 2007). Thus, for many clinical laboratories which do not presently have the capability of performing routine diagnostic polymerase chain reaction (PCR) for respiratory viruses, IF staining could be the diagnostic method of choice.

1.3.4 Antigen Detection

Rapid antigen detection tests are able to provide fast results because they can be applied directly to patient specimens. Often, these tests can be completed in 1 to 3 hours after sample collection. Immunofluorescence assay and enzyme-linked immunosorbent assay (ELISA) are available for the diagnosis of herpes simplex virus, respiratory syncytial virus, influenza A and B, parainfluenza types 1 through 4 and other respiratory viruses to use on nasopharyngeal specimens from pneumonia patients. Unlike ELISA, immunofluorescence assay requires that intact infected cells be prepared in the same manner as for cell culture. ELISA can detect viral antigens that are suspended in solution or associated with inflammatory material. The sensitivity of these methods varies depending on the virus being sought and the specific diagnostic assay (Henrickson *et al.,* 2007).

In the case of bacteria, antigen detection is not performed to detect respiratory pathogens. Instead, it is commonly performed to detect the present of pathogens causing meningitis, for example *Neisseria meningitidis* and Group B streptococcus, as the turnaround time is in a matter of hours, in comparison with the bacterial cultures which take days. Antigen detection tests have several advantages. In the case of virus detection, the use of panels of antibodies to screen clinical specimens for many viruses can be done at one time. In addition, antigen detection assays remain positive for several days to weeks after viable virus can no longer be detected in culture (Chien *et al.*, 2000). This feature is useful in the evaluation of patients who present late after the onset of symptoms or patients who have received prior treatment with antiviral agents. However, overall sensitivity of the viral antigen detection method is lower than viral cultures (Chien *et al.*, 2000; Henrickson *et al.*, 2007) and thus it is recommended that this detection method is used in combination with viral culture for optimal diagnosis of viral infections.

A relatively new adaptation of the antigen detection technique involves using flow cytometry (FCM). It was originally developed as an automated method for measuring the optical or fluorescence characteristics of cells or particles in suspension. Now, almost all microorganisms can be detected in clinical specimens by FCM. Bacteria can be detected as particles in liquid samples in the case of bacteraemia and bacteriuria, and can be identified using polyclonal or monoclonal antibodies that are coated with fluorescent molecules. Due to

their small size, viruses cannot be detected directly using FCM, but can be detected indirectly in infected cells in clinical samples or after inoculation and culture of viruses in cell culture. FCM detects and quantifies viral antigens on the surface of, or within, infected cells. FCM can also detect and quantify viral nucleic acids by in situ hybridization of specific viral nucleic acids in cell suspensions and can simultaneously identify the infected cells by cell phenotyping. This application of FCM has been used for several viruses, including HIV, cytomegalovirus as well as influenza virus (Raoult *et al.*, 2004; Yan *et al.*, 2005).

1.3.5 Serological Detection

Antibodies specific to a pathogen can be detected in patient serum samples to establish an infection in the host. In the case of respiratory viruses, it is commonly done to diagnose RSV and influenza infections (Ostapchuk *et al.*, 2004). For bacteria, usually difficult to culture pathogens are investigated through this method, as in the case of *Mycoplasma* or *Chlamydia*. Serological detection is also useful in the investigation of toxin producing pathogens as culturing of these bacteria are sometimes difficult, as in the case of Group A streptococcus. Both IgM and IgG levels are usually determined in the investigation as the former will determine if it is an active infection and the latter will determined if the patients were ever infected. Though serology

detection for specific antibodies is sensitive, it is not widely used because of the delay inherent in awaiting results on convalescent sera for specific pathogens.

1.3.6 Electron Microscopy

Development of transmission electron microscopy has had a profound impact on our knowledge and understanding of viruses and bacteria. The 1000-fold improvement in resolution provided by electron microscopy (EM) as compared to light microscopy has allowed the visualization of viruses which can be employed in the diagnosis of viral infections on the basis of their morphological differences (Curry *et al.,* 2006). However, the development of molecular techniques that offer greater sensitivity and often the capacity to easily process large numbers of samples means that these techniques have replaced electron microscopy in many areas of diagnostic virology. Hence the role of EM in clinical virology is evolving with less emphasis on diagnosis and more on research, especially the continuing use in the area of investigating unknown, new and emerging agents, such as SARS (Raoult *et al.,* 2004).

In the field of bacteriology, EM is of little use diagnostically, although some bacterial pathogens can be identified in biopsy material processed for EM

examination. Electron microscopy has been used, however, to elucidate the structure and function of many bacterial features, such as flagellae, fimbriae and in the study of bacteriophages (Curry *et al.*, 2006).

1.3.7 Molecular Techniques

1.3.7.1 Polymerase Chain Reaction (PCR)

Molecular diagnostic techniques, such as PCR, have become useful tools for the rapid etiological diagnosis of lower respiratory tract infections. Nucleic acid amplification tests have been evaluated for detecting most respiratory pathogens, and commercial kits are available for some pathogens. PCR has been used as the new gold standard detecting a wide variety of templates across a range of scientific specialties, including virology. Detection of amplicons in conventional PCR relies upon electrophoresis of the nucleic acids in the presence of ethidium bromide, followed by visualization under ultraviolet light. Alternatively, southern blot detection of amplicons using hybridization with labelled oligonucleotide probes or PCR-ELISA detection, used to capture amplicon onto a solid phase using biotin or digoxigenin-labelled primers, oligonucleotide probes or directly after incorporation of the digoxigenin into the amplicon, may be used as post-PCR detection methods (Mackay *et al.*, 2002; Mahony, 2008).

Sensitivity of molecular techniques have been greatly improved by the development of molecular beacons and other fluorescent probes, for example Taqman® probes. Real-time monitoring of PCR amplification as well as quantification of nucleic acids in clinical samples is now possible with this invention. The most commonly used fluorogenic oligoprobes rely upon fluorescence resonance energy transfer (FRET) between fluorogenic labels or between one fluorophore and a dark or "black-hole" non fluorescent quencher (NFQ). This technology, which is highly sensitive, is commercially available as the Taqman® (Applied Biosystems; Perkin-Elmer Corp.) and LightCycler® (Roche Diagnostics Corp.) systems. The advantages of real-time PCR are the rapidity of the assay due to the reduction of amplicon size (30-40 min), the ability to quantify and identify PCR products directly without the use of agarose gels, and the fact that contamination of the nucleic acids can be limited. In addition, real-time PCR has proven cost effective when implemented in a high throughput laboratory, particularly when replacing conventional, culture-based approaches to virus detection (Mackay *et al.*, 2002; Raoult *et al.*, 2004).

Detection of respiratory viruses in clinical samples is important for effective patient management and infection control. Many viruses are involved in respiratory infection and include: influenza viruses A and B, RSV, parainfluenza viruses (PIV) 1-4, adenoviruses (ADV), HRVs, human coronaviruses (hCoV: OC43, 229E, NL-Hong Kong and severe acute

respiratory syndrome coronavirus (SARS-CoV) and hMPV. Many of the infections are indistinguishable by clinical features alone and require rapid laboratory investigation for optimal patient management and infection control. A single tube real-time reverse-transcription PCR has been used extensively in the diagnosis of respiratory viruses even though viral culture used to be the gold standard for laboratory diagnosis (Deffernez *et al.*, 2004; Kuypers *et al.*, 2004; Scheltinga *et al.*, 2005; Henrickson *et al.*, 2007; Mahony, 2008). However, some viruses grow poorly in cell culture, and therefore routine diagnosis is sub-optimal as, for example, with hMPV (van den Hoogen *et al.*, 2004). Furthermore, culture is relatively slow which affects the clinical value, and therefore alternative methods such as real-time RT-PCR are employed (Templeton *et al.*, 2005).

Quantitative PCR, which allows the determination of viral copies, is a useful indicator of the extent of active infection, virus-host interactions and the response to antiviral therapy, all of which can play a role in the treatment regimen selected. The severity of some diseases has been shown to correlate with the viral load, making real-time PCR quantification useful to study not simply only for the presence of a virus but the role of viral reactivation or persistence in the progression of disease. Studies conducted in Tennessee, United States (Perkins *et al.*, 2005) explore the use of real-time reverse transcriptase PCR assays for quantitative assessment of viral load in children affected by respiratory syncytial virus and found this method offered greater

sensitivity, stability after freeze/thaw and lower cost, with great potential for facilitating multi-center studies. In other studies, viral loads have been used as an epidemiological tool where they were able to establish that in RSV infections, the virus load was significantly higher for patients in the lowest (0-6 months) age group compared to older RSV positive children, while the results were the reverse for children with hMPV infections (Kuypers *et al.*, 2004; Kuypers *et al.*, 2005).

1.3.7.2 Sequencing

Together with the development of automated sequences and online databases, sequenced-based identification of microorganisms is becoming a useful and reliable alternative to phenotypic methods of identification. DNA sequencing is the determination of the precise sequence of nucleotides in a sample of DNA. For bacteria, studies of "universal" genes, such as 16S ribosomal RNA gene – have enabled the correct classification of numerous microorganisms however it is not possible to discriminate between all species by just this comparison. "Universal" gene also cannot be used when a higher level of identification is required, such as strain typing in investigations of disease outbreaks, antimicrobial resistance and nosocomial infections. Different gene sequences enable different levels of identification and depending on the gene

used, the genus, species, subspecies or even strain of an organism can be established (Raoult *et al.,* 2004). Strict criteria must be used when interpreting sequence data. A major drawback of online databases is that they are dependent on the quality of the sequences that are submitted by scientists. Inaccurately reported sequences can lead to individual microorganisms being misidentified and even to whole groups of organisms requiring reclassification. In addition, this technique is also limited by the genes which are sequenced.

A more recent adaptation of sequencing for the epidemiological investigation is mass spectrometry. It is an analytical technique that is used for the detection of minute quantities of a molecule in a complex medium. This technique can be used to both measure and analyze molecules under study. It involves introducing enough energy into a target molecule to cause its ionization and disintegration. The resulting fragments are then analyzed, based on the mass/ charge ratio to produce a "molecular fingerprint." A mass spectrometry-based method for detecting single nucleotide polymorphisms has been routinely used as a high-throughput method for genotyping human samples but analysis of base-specific fragmentation patterns of PCR-amplified DNA has recently been studied as a technique for the rapid identification of bacterial isolates and for the detection of specific 16S RNA gene fragments that are amplified from complex environmental samples

(Raoult *et al.*, 2004). This technique shows promise of becoming a standard method for identification of pathogens, strain typing and determination of mutations that are associated with resistance in bacteria and viruses. In a recent study conducted by Liu *et al.* (2005), sequence variation analyses of SARS-CoV isolates demonstrated that common genetic variations in the SARS-CoV genome could be used as "molecular fingerprints" to partition the viral isolates into different lineages, track the transmission of a specific viral lineage, and infer the origin of infection. The advantage is the ability to determine single nucleotide variations throughout the whole genome which therefore makes it a more powerful epidemiological tool than the traditional sequencing done on selected genes.

Other uses of mass spectrometry in the field of microbiology include the genotypic identification of microorganisms including mycobacteria. Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) has been widely applied for the phenotypic characterization of bacterial whole cells, simple cell lysates, or bacterial products such as lipopolysaccharides and proteins (Lefmann *et al.*, 2004). In the case of mycobacteria, the 16S rRNA gene (rDNA) is the most widely accepted gene used for bacterial identification of both cultured and as-yet-uncultured bacteria, with high impact on the discovery of new species of the *Mycobacterium* genus. 16S rDNA sequences constitute one of the largest gene-specific data sets, with constantly increasing numbers of entries in

publicly available databases. In 2002, von Wintzingerode and colleagues described rapid bacterial identification via base-specific cleavage of amplified 16S rDNA and mass spectrometry. Amplification of 16S rDNA signature sequences in the presence of dUTP instead of dTTP was followed by strand separation and uracil-DNA-glycosylase (UDG)-mediated cleavage at each T-specific site. Fragment pattern detection was performed by MALDI-TOF MS and resulted in unambiguous identification of a panel of cultured *Bordetella* spp. and as-yet-uncultured bacteria of anaerobic, organochlorine-reducing microbial consortia.

1.3.8 Microarray

The ability to accurately detect and identify microorganisms that are capable of causing infectious disease has become increasingly important in environmental surveillance, clinical medicine, and bio-defence settings (Palka-Santini *et al.,* 2009). Currently, the predominant techniques used to identify microbial pathogens rely upon conventional clinical microbiology monitoring approaches that are well established and effective but suffer from a number of drawbacks. Standard culture and susceptibility tests permit pathogen identification and antimicrobial susceptibility profiling, but they are laborious, time-consuming, and expensive and require labile natural products (Vora *et al.,* 2004; Templeton *et al.*, 2005; Lee *et al.,* 2008). More

importantly, the biochemical and serologic tests that are routinely utilized for pathogen identification type only to the physiological factors of these organisms. Thus, these assays do not directly provide any information about the potential pathogenicity or virulence of the organism identified. Conventional techniques also do not lend themselves well to managing large numbers of environmental or clinical samples.

Over the past decade the field of clinical microbiology has begun to evolve in response to clinical needs. For bacteriology, automated systems for the detection and identification of microorganisms and for the identification of resistant organisms have been developed. These include automated blood-culture assays and systems for the phenotypic identification of bacteria for example, bioMérieux BacT/ALERT® 3D (www.biomerieux-diagnostics.com) and Becton Dickson BACTEC™ (www.bd.com/ds/productCenter/BC-Bactec.asp). Antibiotic-sensitivity testing and computerized interpretation systems for example, bioMérieux Vitek® 2 (www.biomerieux-diagnostics.com) and have also been developed. Similar developments have also been seen in the virology field where immunoassays (such as the enzyme-linked immunosorbent assay (ELISA which is one of the main detection methods for viruses) have also been extensively automated and standardized over the years (Raoult *et al.*, 2004; Henrickson *et al.*, 2007). These developments, though advanced have not lead to significant changes in microbiology itself, but rather enable more samples to be processed by fewer

personnel and provide increased intra- and inter-laboratory reproducibility (Raoult *et al.*, 2004).

There have also been rapid developments in the genetic detection and identification of microbial pathogens during this period, mainly through nucleic acid amplification by polymerase chain reaction (PCR) (Mahony, 2008). PCR, using universal primers followed by sequencing of the amplification products, has enabled the rapid identification of cultured and uncultured bacteria (Lin *et al.*, 2006), fungi (Klingspor *et al.*, 2009) and especially viruses (Lin *et al.*, 2006; Wong *et al.*, 2007; Lee *et al.*, 2008). These genotypic identification methods utilize molecular biology-based techniques that offer several potential advantages over conventional microbiological approaches used for the identification of pathogens. The direct detection of pathogen-specific DNA or RNA (via PCR or reverse transcription-PCR) addresses the issues of presence and viability without the need for culturing the organism. In this aspect, PCR-based methods offer a distinct advantage for the detection of fastidious and noncultivable organisms (Mahony, 2008; Sloots *et al.*, 2008). These methods are now available in many large university hospital laboratories (Loo *et al.*, 2007; Ong *et al.*, 2007) and are also carried out by many private laboratories (Cockerill *et al.*, 2005). Genetic testing can also be used to identify DNA sequences of pathogens that are associated with antimicrobial resistance (Cockerill *et al.*, 2005). Viral loads also can be determined by PCR and this had been found to be a useful

method for evaluating antiviral therapies (Mahony, 2008; Lee *et al.,* 2009). Finally, molecular methods allow typing of microorganisms, which can help to analyse the epidemiology of outbreaks and identify their sources, as well as distinguishing between relapse and re-infection in the case of persisting microorganisms as a form of infection control (Mahony, 2008).

The development of microarray analysis has been a milestone in several areas of microbiology and in clinical microbiology, microarrays are used for microorganism detection and identification and gene-expression analysis. Microarrays consist of many probes that are discretely located on a solid substrate, such as glass. The probes can be PCR products or oligonucleotides and the "targets" can be PCR products, genomic DNA, total RNA, amplified RNA, complementary DNA, plasmid DNA, bacterial suspensions or clinical specimens (Ye *et al.*, 2001; Wang *et al.*, 2002; Wong *et al.*, 2007).

When PCR is used to detect DNA in clinical specimens, microarrays can then be used to screen and identify the amplified products by hybridization to an array that is composed of pathogen-specific probes. Using broad-range primers, such as those that amplify 16S rRNA gene, a single PCR can be used to detect multiple pathogens simultaneously. DNA microarrays have been used to detect bacteria and viruses and because the number of possible hybridizations is very high, sequencing of a PCR product on an array is possible (Raoult *et al.,* 2004; Wong *et al.,* 2007). In one of the studies, Roth

et al., (2004) developed a diagnostic array of oligonucleotide probes targeting species-specific variable regions of the gene encoding topoisomerases *gyrB* and *parE* of respiratory bacterial pathogens, which allows specific detection of bacterial species. Following this success, the microarray technique showed that it is able to screen, detect and identify multiple pathogens directly from clinical samples without the need for conventional broad-range bacterial PCR method followed by extensive sequencing experiments to identify causative pathogens. Microarray has also been used with success in the detection and genotyping of viral pathogens, including respiratory tract viruses from nasal lavages (Wang *et al.*, 2002; Wong *et al.*, 2007). This is extremely important as many diseases for example pneumonia, have as much as 76% of cases where no pathogens are identified and hopefully with the use of microarray, this issue can be addressed (Nascimento-Carvalho 2001; Sinaniotis, 2004). In addition, microarray can also assist in the diagnosis of novel infections where the etiological agent is unknown or in cases where large numbers of other infectious diseases resulting in co-infections occurs (Wang *et al.*, 2003; Wong *et al.*, 2007).

The application of microarray technology for microbial diagnostics is a field in the stage of dynamic development. Oligonucleotide probe sets were used to specifically detect different genotypes of rotavirus, *Listeria* as well as *Cryptosporidium* isolates (Bodrossy *et al.*, 2004). 40mer oligoprobes based on the 16S rRNA gene were used to detect 20 common bacterial species of the

human intestinal flora. An Affymetrix GeneChip with over 30,000 16S rRNA targeting oligo probes was used to identify culture collection species and subsequently to characterize populations of airborne bacteria at the level of higher phylogenetic taxa (Bodrossy *et al.,* 2004). In a recent study conducted by Lin *et al.* (2006), the group demonstrated the use of resequencing DNA microarrays for broad-spectrum respiratory tract pathogen identification. This platform demonstrated the ability to simultaneously detect respiratory pathogens in clinical samples without requiring the design of pathogen-specific PCR primers or fixed hybridization patterns. Another potential and important use of the microarray technology in the diagnosis of an infection is to assist in the differentiation between colonization of a microorganism (especially bacteria) and a true infection. By using quantitative PCR to establish a cut-off level, one can therefore use this method to first screen the patient samples for potential pathogens, followed by identifying the true causative agent.

1.4 Disease Severity in Pneumonia

Infection is one of the leasing causes of human mortality and morbidity, with much of the burden falling on children. Susceptibility to infections arises due to the complex interaction of environment, host genetic factors as well as pathogenic factors of the infectious microorganisms (Burgner *et al*., 2006). However, relationships between these factors and disease severity are poorly defined, though many studies have attempted to understand the underlying causes with controversial results (Fodha *et al*., 2007). For host factors, studies have indicated that young age (6 months), prematurity, low-birth weight, and chronic diseases may be associated with disease severity; while environmental factors for example air pollution (including environmental tobacco smoke) and over-crowding could also increase the risk of severe diseases (.e.g. pneumonias) (Singh *et al*., 2005; Fodha *et al*., 2007). On the other hand, viral factors associated with disease severity are not understood sufficiently. In the case of respiratory viral infections, published reports disagree as to whether or not there is a difference in the pathogenicity of viral type, viral strains as well as viral load and their association with more severe illness.

In term of respiratory viral infections, RSV has been known to cause severe lower respiratory tract infections which include bronchiolitis and pneumonia, especially in infants and young children (Savon *et al*., 2006; Fodha *et al*., 2007; Mathisen *et al*., 2010). RSV isolates which can be divided into two

subgroups A and B circulate independently in the human population. A number of studies which compared severity of infants infected with RSV A and RSV B had concluded that infections with RSV A generally are associated with more severe illness than RSV group B (Peret *et al*., 2000; Savon *et al*., 2006). However, other authors had shown that disease severity was not associated significantly with RSV subgroups; rather, it was likely to be determined by an interplay between host and RSV viral load (Fodha *et al*., 2007; Gerna *et al*., 2008). These observations were also noted for hMPV where a high hMPV viral load correlated with disease presentation, whereas the overall clinical and socioeconomic burden caused by hMPV genotypes was similar (Gerna *et al*., 2007; Bosis *et al*., 2008). As for HRV, one of the most common causes of asthma exacerbation, study conducted by van Elden *et al.*, (2008) concluded that severity of lower respiratory tract symptoms were not related to viral load in patients. Thus, the issue of disease severity remains controversial and further study is clearly needed in this important area in order to derive better patient management strategies.

1.5 Respiratory Samples Used for Pathogen Detections

Different types of specimen are available for the diagnosis of respiratory virus infections. Nasopharyngeal respiratory secretions collected from infants with bronchiolitis by direct aspiration (nasopharyngeal aspirate, NPA) are a valuable resource for the study of virus dynamics and local inflammatory as well as the diagnosis of respiratory pathogens (Semple *et al*., 2007). NPA, though has been considered to be the best sampling technique, is however more invasive and results in significantly more distress of the infants than nasal swabs (Meerhoff *et al*., 2010). As such, many studies were conducted to look for alternative samples in replacement of NPA (Lambert *et al*., 2008; Meerhoff *et al*., 2010). This was made possible as the use of more sensitive molecular diagnostics allows for the collection of a less invasive clinical specimen while increasing sensitivity of diagnosis for viruses and other fastidious organisms. Generally, specimens being considered were nasopharyngeal swabs, nose-throat swabs and nasal swabs. Study conducted by Lambert *et al.,* (2008) which compared nose-throat swab with NPA noted as sensitivity of 91.9% for the detection of Influenze A and 93.1% for the detection of RSV for the former collection method. This was also observed by Meerhoff *et al.,* (2010) which detected the sensitivity of nasal swab for RSV detection to be 51% as compared to 100% for NPA and 75% for HRV as compared with 97% for NPA. However, both studies concluded that, for community-based studies and surveillance purposes, both the nasal and nose-

throat swabs can be used, though sensitivity is lower than the NPA. As such, children who are present in a hospital for severe infections should still have their NPAs collected for the purpose of differential diagnosis.

1.6 Current Limitations in The Laboratory Detection of Respiratory Viruses

Clinical microbiology evolved in response to clinical needs and there have been many advances in this area during the past decade. For example, automated systems for the detection and identification of microorganisms; automated blood-culture assays and systems for the phenotypic identification of bacteria had been developed. However, these developments have not led to significant changes in the microbiology itself but rather enable more samples to be processed by fewer personnel and provide increasing intra- and inter-laboratory reproducibility (Raoult *et al.,* 2004). Most importantly, these developments are targeted mainly to the area of bacteriology and not virology.

The direct diagnoses of viruses in many clinical laboratories are still based on conventional methods such as isolation by cell culture and antigenic detection, especially when they are new and novel viruses. Even though these methods can be effective and often complementary, they are either too laborious, time consuming or not sensitive and specific enough for diagnosis. In certain cases, viruses may grow poorly in cell culture, and therefore routine diagnosis is sub-optimal as in the case of hMPV (Bellau-Pujol *et al.,* 2005; Templeton *et al.,* 2005). However, it is important for the detection of viruses in clinical samples as part of effective patient management, especially since the possibilities for antiviral treatment have increased. In addition, the

need for more rapid diagnoses in the face of new threats from infectious microorganisms, especially with viruses involved in respiratory infection, is important for infection control purposes. These viruses namely: influenza viruses A and B, RSV, parainfluenza viruses (PIV), HRVs, human coronaviruses (hCoV), adenoviruses (ADV), acute respiratory syndrome coronavirus (SARS-CoV) and hMPV are usually indistinguishable by clinical features alone and require laboratory investigation for their diagnosis (Raoult *et al.,* 2004; Templeton *et al.,* 2005).

Nevertheless, detection of viral respiratory agents has improved tremendously with the development of new molecular techniques. These developments allowed rapid genetic detection and identification mainly through nucleic acid amplification by PCR. With the introduction of real-time PCR, it is also now possible to determine viral loads which might be a useful method for evaluating antiviral therapies in conjunction with clinical responses (Niesters, 2004; Raoult *et al.,* 2004). However, most of these molecular methods are mainly performed in research laboratories rather than in clinical microbiology laboratories and reasons include: difficulties in determining primers and probes for the detection of specific viruses, lack of standardisation between laboratories and in the case of quantifying viral load; difficulties in the normalization of patient specimens (Gueudin *et al.,* 2003; Dimech *et al.,* 2004; Raoult *et al.,* 2004). Despite the problems encountered for PCR-based methods, it is possible to overcome these obstacles with the

introduction and use of standardised materials, participation in international quality control programs and with the introduction of a universal internal control throughout the whole procedure for accuracy in the results generated (Niesters, 2004).

There have been substantial changes in the role of the clinical microbiology laboratory over the past decade. The ongoing technological revolution has rapidly transformed research, diagnostic and therapeutic tools and with newer technologies being developed, for example microarrays, mass spectrometry and flow cytometry, the era of clinical molecular microbiology in the very near future is inevitable.

1.7 Aims and Objectives of the Present Study

The features of childhood community acquired pneumonia vary between countries and individuals. Despite numerous studies conducted to investigate factors contributing to disease severity in children with ALRTIs worldwide, there is still no clear indication of which factor causes severe pneumonia. Thus, the main objective was to investigate viral factors contributing to disease severity in paediatric patient pneumonia. Utilizing nasopharyngeal washes collected from a birth cohort study in Indonesia, we aimed to investigate disease severity by determining:

(1) the frequency of known agents causing pneumonia.
(2) whether or not there were new agents causing pneumonia.
(3) the frequency of co-infections in pneumonia.
(4) the viral loads of specific pathogens in the nasopharyngeal samples collected from patients with pneumonia.
(5) the presence of any specific virulence strain of a particular pathogen causing pneumonia.
(6) the seasonality of any specific pathogens detected.

It was hypothesized that specific viral infections, increased viral loads, and the presence of specific viral strains as well as viral co-infections could result in more severe pneumonia in these infected individuals.

CHAPTER 2 – COLLECTION AND PREPARATION OF CLINICAL SAMPLES

2.1 Patient Recruitment

This longitudinal birth cohort study was conducted at two sites in Bandung Kota, West Java district of Indonesia, situated at an elevation of 768 meters above sea level, by Professor Eric Simoes, Cissy Kartasasmita and their research team. The study was conducted in a peri-urban community: Cikutra (population 53,000) and a semi-rural community: Ujung Berung (population 42,000) (Figure 2.1). For this cohort study, all babies born in both sub-communities were prospectively enrolled for this study after obtaining informed consent from parents or caretakers. Children under 59 months were also recruited for this study. These subjects were enrolled from February 1999 and followed up until May 2001.

Female community health workers (kaders) who lived in the sub-community in close proximity to the households of the study infants made weekly home visits for the duration of the study. They interviewed the mother, and if the child had a cough or difficult breathing, measured the respiratory rate, examined the chest for lower chest wall indrawing and listened for audible wheeze. Lower respiratory tract infection was determined using standard WHO case definitions (Mulholland *et al.*, 1992; WHO, 1995). Tachypnoea was defined as a respiratory rate ≥ 60/minute for infants 1 week to 2 months, ≥ 50/minute for infants 2 to 11 months, and ≥ 40/minute for children 12 to 59

Resource : Microsoft Expedia Maps

Figure 2.1. Map of Indonesia with reference to Bandung, the site where the study was conducted.

months. The WHO classifies a child with tachypnoea and no chest indrawing as having mild pneumonia. To assess severity of pneumonia, a child with lower chest wall indrawing is classified as having severe pneumonia (with or without tachypnoea) and very severe pneumonia if there is either severe lethargy, difficulty to arouse, or general danger signs (Table 2.1). Kaders were trained using standard WHO materials translated into Bahasa Indonesia with a refresher course every 6 months.

Children with tachypnoea, lower chest wall indrawing or audible wheeze were escorted to the private practitioner for assessment by a physician where they were examined for signs of lower respiratory tract disease, and a nasal wash was obtained for testing if they had any of the 3 signs. Nasopharyngeal specimens were collected by instilling 5-10 mL of normal saline solution into the child's nasopharynx and collecting the wash material in a test tube or by aspiration. This was transferred into sterile containers stored at 4°C and transported twice a day (usually within 1-2 hours of collection) on ice to the research laboratory at the medical research unit, RS Hasan Sadikin Hospital, Padjajaran University, Bandung, Indonesia. Following standard WHO protocols, a child classified as having pneumonia was administered either cotrimoxazole or amoxicillin for 5 days. Children with wheezing were

(a)

Severe pneumonia	Severe chest indrawing or fast breathing
Very severe pneumonia	Not feeding Convulsions Abnormally sleepy or difficult to wake Fever/low body temperature Hypopnea with slow irregular breathing

(b)

Mild pneumonia	Fast breathing
Severe pneumonia	Chest indrawing
Very severe pneumonia	Not able to drink Convulsions Drowsiness Malnutrition

Table 2.1. (a) Assessment of severity of pneumonia in infants below two months old. (b) Assessment of severity of pneumonia in children age 2 months to 5 years old. (Adapted from WHO, 1991)

administered with albuterol through a metered dose inhaler with spacer or a neublizer. Children with persistent lower chest wall indrawing were referred to a hospital for admission. All children with pneumonia were followed up by kaders every 2 days until they were well using a specific follow-up form to define the clinical presentation and course of lower respiratory tract infection. If after 2 days there was no improvement, the sick child was brought back to the physician for re-evaluation. If needed, subsequent nasal aspirates were collected (Figure 2.2).

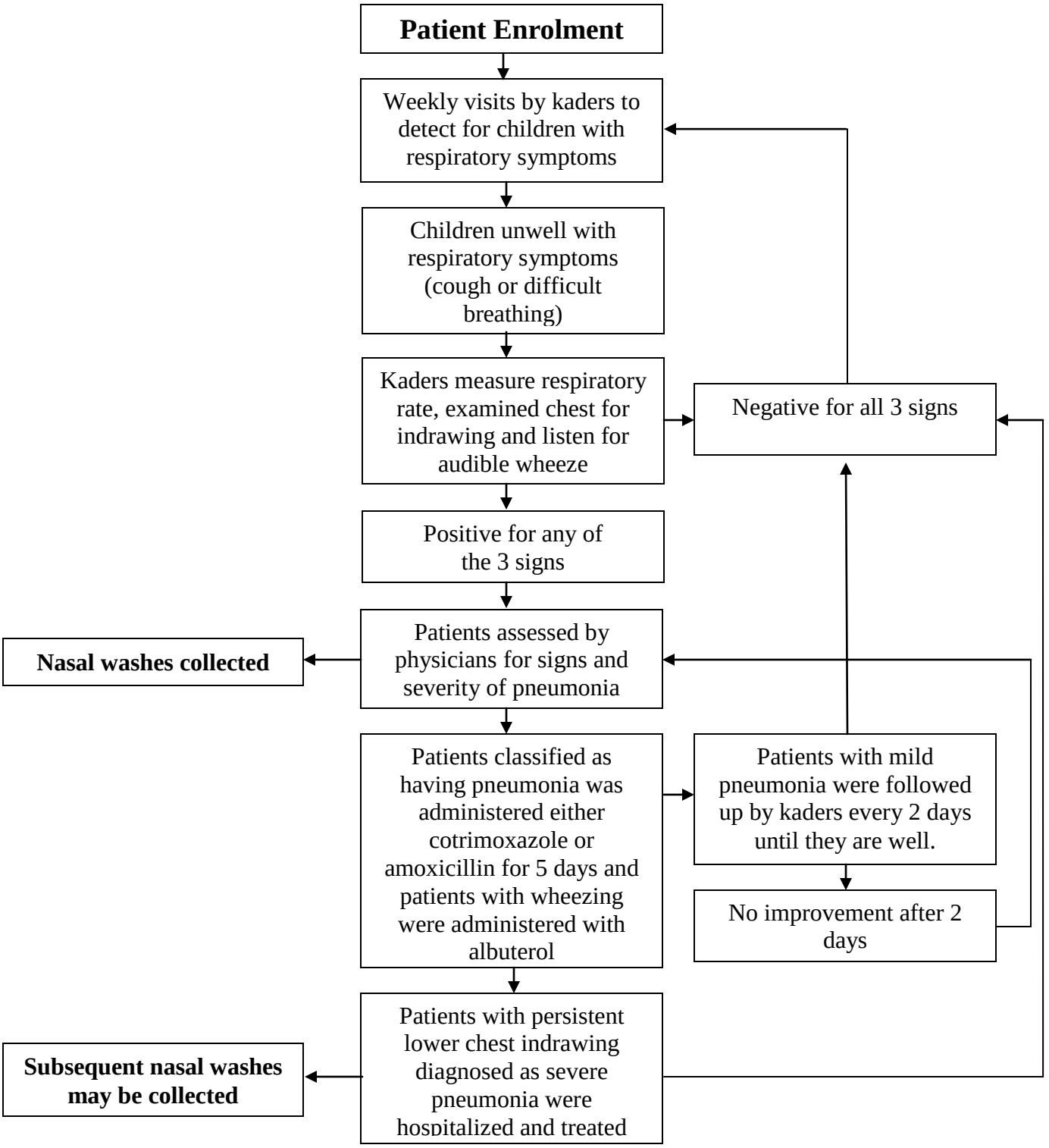


Figure 2.2. Flowchart on patient management and sample collection.

In the 28 months of the study, a total of 2014 infants and children were enrolled for this study. Of these, 352 episodes of pneumonia were sampled from 256 patients and samples were collected and subjected to real-time RT-PCR.

2.2 Clinical Samples

A total of 352 clinical specimens from 256 patients were obtained from an Indonesian cohort population collected during February 1999 to May 2001 by Professor Eric Simoes and his research team. All patients enrolled were between the ages of 0 to 59 months, and demonstrated specific clinical signs of respiratory illnesses. Lower respiratory tract infection was determined using the WHO case definitions (Mulholland *et al.*, 1992; WHO, 1995) and pneumonia was suspected for all cases. Nasopharyngeal washings were collected from these patients and stored in RNAzol (Leedo Medical Laboratories, Inc., Friendswood, Tex.) at -80°C.

2.3 Nucleic Acid Isolation

Half of nasopharyngeal washings from patients were centrifuge in sterile, RNase free Eppendorf tubs for 2 minutes at 14,000g. The resulting cell pellet was re-suspended in 750 µLof Trizol™ (Invitrogen, Life Technologies, USA) and frozen at -86°C. RNA was extracted according to manufacturer's instructions and re-suspended in RNA storage solution (Ambion, USA) and kept at -80°C until use. For extraction, chloroform was added into the sample and incubated followed by centrifugation. The sample was then separated into the RNA in the upper aqueous phase and the DNA in the lower organic phase. The upper aqueous phase was carefully transferred into a fresh micro-centrifuge tube and an equal volume of 70% ethanol was added. The sample was subsequently purified using the Qiagen RNA Mini Kit (Qiagen, Germany) following the manufacturer's instructions.

2.4 Climatic Data

Daily climatic data, minimum and maximum temperatures, relative humidity, and rainfall, was obtained from the meteorological office in Bandung on a monthly basis.

CHAPTER 3 – EVALUATION AND USE OF MICROARRAY ASSAYS TO DETECT RESPIRATORY PATHOGENS IN NASOPHARYNGEAL SAMPLES FROM A PAEDIATRIC COHORT

3.1 Introduction

Although PCR-based assays are exquisitely sensitive, accurate, and relatively rapid at detecting pathogens requiring only minimal amounts of material for identification (Henrickson *et al.,* 2007; Mahony, 2008), their use also introduces a new set of drawbacks. A successful identification of the pathogen depends almost entirely on appropriately chosen primer sets. All PCR-based testing requires a prior knowledge pertaining to the genomic identity of the potential pathogen. Additionally, technical challenges make it difficult to simultaneously address the issues of identification, potential pathogenicity, and antimicrobial susceptibility. In an attempt to circumvent these drawbacks for pathogen detection, researchers have developed multiplex PCR assays that amplify up to six unique diagnostic regions for a single organism or identify multiple different organisms within a single experimental reaction (Bellau-Pujol *et al.,* 2005; Kuypers *et al.,* 2006; Brittain-Long *et al.,* 2008). Although these assays are a marked improvement over traditional PCR-based assays, multiplex assays are still limited in the scope and overall output that are now necessary for pathogen identification. As such, there remains a critical need for advanced diagnostic systems that can rapidly screen clinical and environmental specimens without bias for the presence of pathogenic organisms. Nucleic acid hybridization to DNA microarrays is an experimental approach that has demonstrated great promise in addressing this need (Lin *et al.,* 2006; Wong *et al.,* 2007; Lee *et al.,* 2008).

The advantage of microarray-based detection is that it can combine powerful nucleic acid amplification strategies with the parallel screening capability of microarray technology, resulting in a high level of sensitivity, specificity, and throughput capacity. In an attempt to address the bottleneck created by specific PCR-based amplification techniques, the Microarray team in the Genome Institute of technology (GIS) in Singapore created an original oligonucleotide array using Nimblegen array synthesis technology to detect up to 35 RNA viruses using 40-mer probes tiled at an average 8-base resolution across the full length of each genome with a novel robust algorithm, Pathogen Detection Algorithm (PDA). This PDA can accurately predict PCR bias during DNA amplification and can be used to improve PCR primer design. It can also be used as a powerful statistical concept for inferring pathogen identification from probe recognition signatures (Wong *et al.,* 2007). Thus, to assess the clinical utility of the pathogen prediction platform, 36 nasal washed specimens from the present study were analysed and compared with results obtained for real-time RT-PCR for the presence of human metapneumovirus (hMPV), respiratory syncytial virus (RSV), both A and B genotypes.

Although detection of pathogens using microarray technology is a relatively young field, (Wong *et al.*, 2007) a number of different platforms and approaches have been described, each with important attributes. For example, the array described by Wang *et. al.* (2002) is based on probes designed to

recognize the most conserved viral domains, facilitating the detection of a taxonomic fingerprint that provides powerful clues to viral identity with minimal probe usage. Lin *et. al.* (2006), on the other hand, described a probe-dense re-sequencing array capable of detecting a smaller set of predefined pathogens, but with higher detection specificity, including the ability to discern highly related subtypes.

3.2 Materials and Methods

With the guidance of her local supervisor, A/Prof Martin Hibberd, the author worked and liaised with the Microarray team in Genome Institute of Technology (GIS), Singapore on the selection of respiratory pathogens for the pathogen chip design. The platform was eventually developed by Wong *et al.*, (2007) in the Microarray team, GIS for evaluation.

Briefly, complete genome sequences of 35 clinically relevant human viruses (Appendix 1) were downloaded from the NCBI Taxonomy Database (www.ncbi.nlm.nih.gov) and used to generate 40-mer probe sequences to tile across each genome, overlapping at an average 8-base resolution. Seven replicates of each probe were synthesized at random positions on the microarray using Nimblegen proprietary technology (Nuwaysir *et al.*, 2002). For quality control purposes, 10,000 random sequences probes with 40-60% GC content were included to assess background signal levels. Additional controls included 400 probes to human immune genes (positive controls) and 162 probes to a plant virus, PMMV (negative control). In total, 390,482 probes were synthesized on the array.

To assess the clinical utility of the pathogen prediction platform, 36 nasal wash specimens were obtained from paediatric patients of the Indonesian birth cohort, of which 14 were suffering from severe pneumonia and 22 with

mild pneumonia. All specimens were analyzed by microarray in a blind fashion.

Briefly, RNA was reverse transcribed to cDNA using tagged random primers. The original primer A1 was 5'-GTTTCCCAGTCACGATANNNNNNNNN-3'; and the AES-optimized primer A2 was 5'-GATGAGGGAAGATGGGGNNNNNNNNN-3'. The cDNA was then amplified by random PCR, fragmented, end-labelled with biotin, hybridized onto the microarray and stained. Microarrays were scanned at 5um resolution using an Axon 4000b scanner and Genepix 4 software (Molecular Devices, Sunnyvale, CA, USA). Signal intensities were extracted using Nimblescan 2.1 software (NimbleGen Systems, Madison, WI, USA). Using an automated script (personal communication with George and Vega), median signal intensity and standard deviation from the seven replicates of each probe were calculated. The probe signal intensities were sorted by genome and arranged in sequence order, then reformatted into CT format for graphical viewing of signal intensities in Java Treeview. In parallel, the probe median signal intensities were analysed using a further software development "PDA" to determine which pathogen was present, and the associated confidence level of prediction. Detailed protocol can be obtained in Appendix 2.

3.3 Results

The microarray used in the present study was designed to detect up to 35 RNA viruses using 40-mer probes tiled at an average 8-base resolution across the full length of each genome (53,555 probes; Appendix 3). Together with seven replicates for each viral probe, and control sequences for array synthesis and hybridization, the array contained a total of 390,482 probes. In an initial study, virus samples purified from cell lines, reverse-transcribed and PCR-amplified with virus-specific primers (instead of random primers) were used (Wong *et al.*, 2007). This allowed array hybridization dynamics in a controlled fashion to be studied, without complexity of cross-hybridization from human RNA and random annealing dynamics, which occur with random primers. The findings were then applied to clinical samples amplified using random primers. An example of the hybridization pattern of RSV serotype 1 can be seen in Figure 3.1.

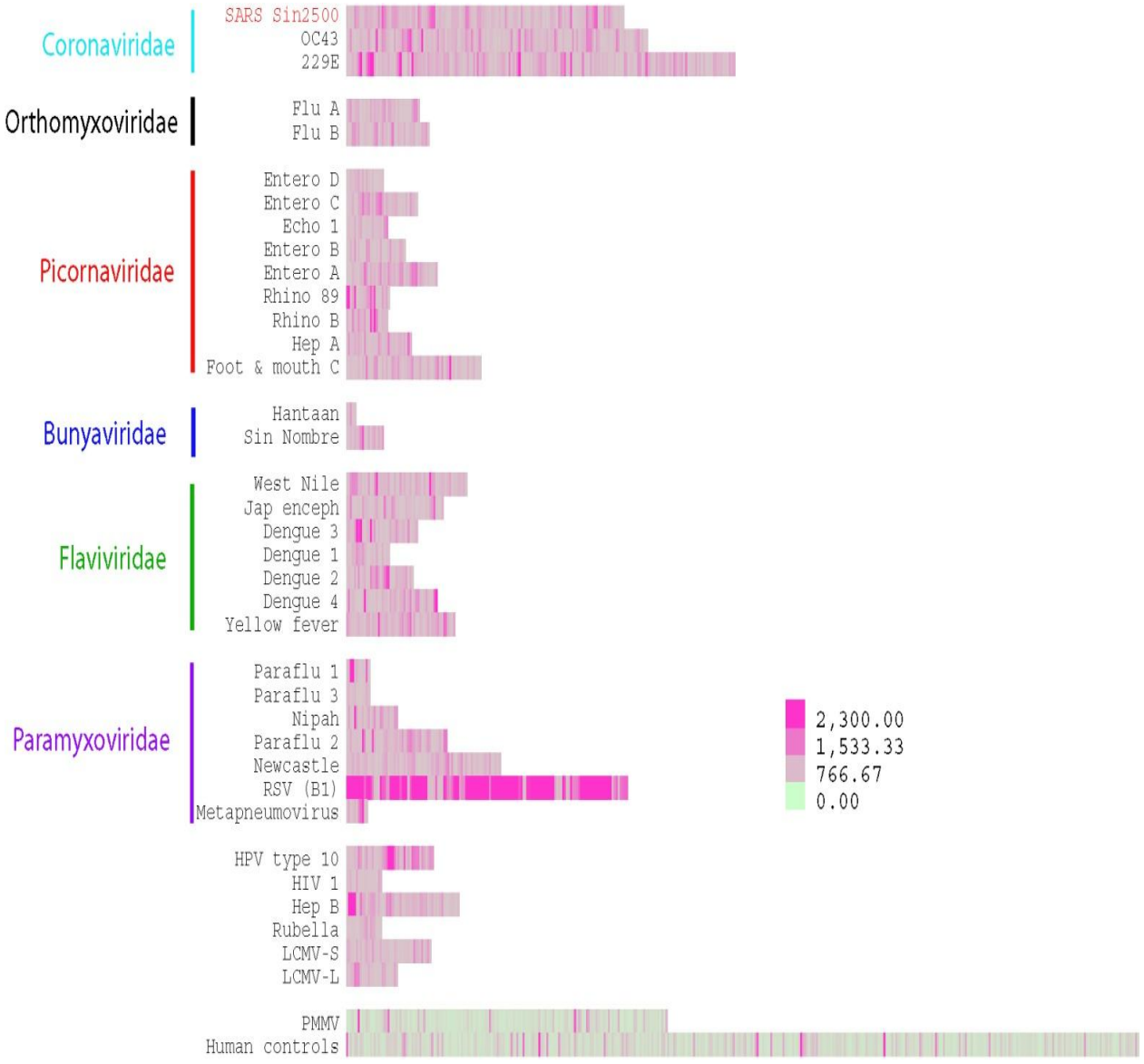


Figure. 3.1. Heatmap of microarray probe signal intensities. Cells corresponding to probes are aligned in genomic order and coloured according to the signal intensity-colour scales shown for RSV isolate.

To understand the relationship between hybridization signal output and annealing specificity, all probe sequences were first compared to each viral genome using two measures of similarity: probe hamming distance (HD) and maximum contiguous match (MCM). HD measured the overall similarity distance of two sequences, with low scores for similar sequences. MCM measured the number of consecutive bases that are exact matches, with high scores for similar sequences. Using these optimal HD and MCM thresholds to guard against cross- hybridization, all probes were binned into specific "recognition signature probe sets" (r-signatures) most likely to specifically detect a given pathogen. For each of the 35 pathogens, the r-signature of the genome was defined and represented on the array. Thus, each pathogen's r-signature comprised tiling probes derived from its genome sequence, as well as cross-hybridizing probes derived from other pathogens. According to these criteria, a given probe could belong to multiple different r-signatures, thereby maximizing probe-level evidence for pathogen detection.

As part of the research in this thesis, the clinical utility of the pathogen prediction platform was evaluated using 36 nasal washes specimens from the 352 specimens collected from the cohort studied and analysed according to the workflow illustration (Appendix 4) in a blinded trial. All 36 specimens were first analyzed for the presence of human metapneumovirus (hMPV), respiratory syncytial virus (RSV), both A and B genotype using real-time reverse-transcriptase PCR (RT-PCR) before microarray analysis. Twenty-one

specimens were tested positive for one or more viruses, while 15 were PCR-negative for all three viruses.

Assay performance of the platform was carried out first with 16 patients diagnosed with either hMPV or RSV B by real-time RT-PCR (Table 3.1). As the RSV A full-genome sequence had not been published at the time of this present investigation, the array was not designed to specifically detect this virus.

Microarray correctly detected the presence of hMPV or RSV B in 13 out of 16 samples, corresponding to an assay specificity of 100%, sensitivity of 76%, and diagnostic accuracy of 94% (Table 3.1). All false negative samples (patients 374, 841, 892, and 924) had Ct values > 33.5, which is near the detection limit of real-time PCR, and thus perhaps beyond the range of detection by microarray.

In the next assessment of array performance samples from the group of patients that were PCR-positive for RSV A (n=7) and PCR-negative for all other tested viruses (n=15) were tested. Of the 22 samples, only two positive results were recorded for this group by the microarray. Both of these results were for RSV B. Interestingly, both RSV B positive results corresponded to high-titre RSV A, suggesting that certain probe sets can detect the presence of related, but unspecified, viruses. Analysis of the published RSV A partial

Table 3.1 Comparison of microarray and real-time RT-PCR performance in detecting RSV B or hMPV from nasopharyneal samples.

Patient ID	Array	WKL	*P* value	PDA diagnosis	RT-PCR diagnosis	RT-PCR Ct value	Virus copy no./μL
122	35887	20.87	2.47×10^{-29}	hMPV	hMPV	24.8	5.0×10^{4}
133	71180	22.33	6.93×10^{-62}	hMPV	hMPV	25.1	4.0×10^{4}
165	66691	16.95	3.49×10^{-4}	hMPV	hMPV	27.9	3.9×10^{3}
254	70935	25.02	2.87×10^{-39}	hMPV	hMPV	22	5.4×10^{5}
769	73067	24.62	3.70×10^{-52}	hMPV	hMPV	25.7	2.5×10^{4}
832	73068	13.52	4.54×10^{-6}	hMPV	hMPV	28.2	3.1×10^{3}
892	68359			ND	hMPV	34	27
324	35259	20.61	3.55×10^{-94}	RSV B	RSV B	21.4	3.0×10^{6}
355	35662	18.00	2.97×10^{-40}	RSV B	RSV B	20.3	6.7×10^{6}
374	66695			ND	RSV B	34.1	500
378	70933	13.82	7.77×10^{-17}	RSV B	RSV B	23.9	5.4×10^{5}
412	35890	19.66	2.42×10^{-49}	RSV B	RSV B	23.5	6.9×10^{5}
483	36053	12.17	1.47×10^{-12}	RSV B	RSV B	24.8	2.9×10^{5}
924	66703			ND	RSV B	33.7	630
337	71192	21.73	3.49×10^{-14}	RSV B	RSV B	32.1	1.1×10^{5}
841	73070	22.66	4.21×10^{-50}	RSV B	RSV B	20.9	4.4×10^{6}
					hMPV	35.4	8

WKL: weighed Kullback-Leibler (KL) Divergence, a modified KL divergence; PDA: Pathogen Detection Algorithm; hMPV: human metapneumovirus; RSV B: respiratory syncytial virus B; ND: hMPV or RSV B not detected. Genome Biol. 2007; 8(5): R93. Published online 2007 May 28. doi: 10.1186/gb-2007-8-5-r93.Copyright © 2007 Wong *et al.*; licensee BioMed Central Ltd.

genome sequence (923 bp, Genbank ID: AF516119) revealed that seven probes on the microarray had 100% identity to RSV A. Thus, an "RSV A r-signature" comprising of these seven probes was created by the Microarray team and this enabled the detection of RSV A by microarray in four out of the seven patients samples positive for RSV A by real-time RT-PCR (i.e. patients 414, 832, 913 and 924) (Table 3.2).

Although the performance of this small r-signature was not as robust as the other virus r-signature, it did allow the possibility of a "viral discovery" approach to detect viruses at family or genus level that were related to those species already represented on the microarray. This was made possible as probes were binned into family- or genus-level r-signatures by relaxing similarity criteria and selecting probes common to genome sequences within families and genera for the piconaviridae family, paramyxoviridae family, rhinovirus genus (HRV) and pneumovirus genus (inclusive of RSV and hMPV).

After modifications performed by the Microarray team, all 36 samples were re-analysed in the present investigation using the family specific approach looking for "relaxed" r-signatures. From this analysis, pneumoviruses (RSV A, RVS B and/or hMPV) were detected in 17 specimens (1 false positive, patient 283), and additionally nine HRV were also detected (Table 3.2). As HRV was a novel discovery, all 36 were re-screened by real-time RT-PCR for the

Table 3.2 Comparison of microarray and real-time PCR performance in detection of pathogen genera (HRV, pneumovirus) in 36 clinical samples.

Patient ID	Array	WKL	*P* value	PDA genus diagnosis	PCR diagnosis	PCR Ct value	Virus copy no.
111	35915			ND	ND		
122	35887	20.87	2.47×10^{-29}	Pneumovirus	Pneumovirus	24.8	5.0×10^{4}
133	71180	22.33	6.93×10^{-62}	Pneumovirus	Pneumovirus	25.1	4.0×10^{4}
165	66691	16.95	3.49×10^{-4}	Pneumovirus	Pneumovirus	27.9	3.9×10^{3}
185	66696			ND	ND		
254	70935	25.02	2.87×10^{-39}	Pneumovirus	Pneumovirus	22	5.4×10^{5}
261	66697			ND	ND		
283	63781	23.99	2.28×10^{-25}	Pneumovirus	HRV	28.3	6.1×10^{4}
		14.07	4.66×10^{-11}	HRV	ND		
312	66701			ND	Pneumovirus[†]	33.7	44
321	71006			ND	Pneumovirus[†]	31.1	340
324	35259	20.61	3.55×10^{-94}	Pneumovirus	Pneumovirus	21.4	3.0×10^{6}
331	66698			ND	HRV	31.7	3.6×10^{3}
337	71192	21.73	3.49×10^{-14}	Pneumovirus	Pneumovirus	26.2	1.1×10^{5}
		8.3	1.92×10^{-4}	HRV	HRV	29.1	3.1×10^{4}
355	35662	18.00	2.97×10^{-40}	Pneumovirus	Pneumovirus	20.3	6.7×10^{6}
368	66702			ND	ND		
374	66695			ND	Pneumovirus	34.1	500

Patient ID	Array	WKL	*P* value	PDA genus diagnosis	PCR diagnosis	PCR Ct value	Virus copy no.
378	70933	13.82	7.77×10^{-17}	Pneumovirus	Pneumovirus	23.9	5.4×10^{5}
393	71189	25.41	1.15×10^{-18}	HRV	HRV	30.2	2.1×10^{5}
412	35890	19.66	2.42×10^{-49}	Pneumovirus	Pneumovirus	23.5	6.9×10^{5}
414	71025	49.91	1.18×10^{-65}	Pneumovirus†	Pneumovirus†	22.3	3.9×10^{5}
					HRV	33	2.6×10^{3}
461	66699			ND	ND		
478	71027			ND	Pneumovirus†	34.8	18
483	36053	12.17	1.47×10^{-12}	Pneumovirus	Pneumovirus	24.8	2.9×10^{5}
554	70997	78.55	4.59×10^{-120}	HRV	HRV	23.5	1.5×10^{6}
573	66700	38.09	6.26×10^{-22}	HRV	HRV	22.2	3.6×10^{6}
639	71182	9.23	7.91×10^{-6}	HRV	ND		
699	71007			ND	ND		
769	73067	24.62	3.70×10^{-52}	Pneumovirus	Pneumovirus	25.7	2.5×10^{4}
818	70927	10.40	1.63×10^{-8}	HRV	HRV	34.2	1.2×10^{3}
832	73068	13.52	4.54×10^{-6}	Pneumovirus	Pneumovirus	28.2	3.1×10^{3}
		40.43	1.73×10^{-36}	Pneumovirus†	Pneumovirus†	23.8	1.2×10^{5}
841	73070	22.11	6.80×10^{-50}	Pneumovirus	Pneumovirus	20.9	4.5×10^{6}
						35.4	8
					HRV	29.2	3.3×10^{4}
853	66690			ND	ND		

Patient ID	Array	WKL	*P* value	PDA genus diagnosis	PCR diagnosis	PCR Ct value	Virus copy no.
859	71188	72.17	1.42×10^{-128}	HRV	HRV	24.5	2.8×10^{6}
892	68359	12.43	5.77×10^{-5}	HRV	Pneumovirus	34	27
					HRV	32.3	4.2×10^{3}
913	71028	40.67	1.60×10^{-50}	Pneumovirus†	Pneumovirus†	19.1	4.7×10^{6}
924	66703	12.79	2.56×10^{-6}	Pneumovirus†	Pneumovirus†	31.5	250
					Pneumovirus	33.7	630

WKL: weighed Kullback-Leibler (KL) Divergence, a modified KL divergence; PDA: Pathogen Detection Algorithm; †RSV A patient samples; HRV: human rhinoviruses; ND: none detected. Genome Biol. 2007; 8(5): R93.Published online 2007 May 28. doi: 10.1186/gb-2007-8-5-r93.Copyright © 2007 Wong *et al.*; licensee BioMed Central Ltd.

presence of HRV. Primers and probes obtained from Contoli *et al.*, (2006) targeting a conserved portion within the 5' non-coding region were used for detecting HRVs from clinical samples. In total 11 out of 36 specimens were found to be positive for HRV. All nine HRV positives by microarray were confirmed by real-time RT-PCR except for one (patient 639). Similarly, the pneumovirus genus detected in patient 283 could not be verified by real-time RT-PCR. These findings were intriguing given that the HRVs have over 100 serotypes with different genomic diversities, thus making the detection by PCR notoriously difficult.

Though the microarray identified the majority of HRV and RSV A samples using the genus-level r-signatures, it failed to detect three samples positive for HRV (patients 331, 414, 841) and three positive for RSV A (patients 312, 321, 478) by real-time RT-PCR . In general, these samples had Ct values >32, suggesting a detection threshold close to but lower than that of real-time RT-PCR. Even then, there were a number of accurate discoveries in the 30-35 Ct range suggesting detection variability when the real-time RT-PCR Ct level was above 30. Notably, the microarray correctly detected the presence of co-infecting pathogens in two samples (337 and 832), demonstrating the unique potential of this microarray platform to reveal mixed infectious status. Most importantly, for this study, it also acted as a screening tool for our patient samples, which prompted us to investigate the entire cohort not only for the detection of hMPV, RSV A and RSV B but also for the presence of HRVs.

3.4 Discussion

The microarray used for the present study represents a blend of two concepts, integrating a probe tiling approach for substantial genomic coverage (though with lower probe density than resequencing arrays), with a taxonomy-based strategy for binning probes into pathogen recognition signatures. Thus, our analytical output includes both family- and genus-level predictions (for r-signatures) restricted to conserved probes as well as species-specific predictions (for r-signatures composed of conserved and unique probes). Indeed, this capability allowed us to detect and accurately identify novel viruses in clinical samples.

Compared to real-time RT-PCR results, the microarray platform used in the present study identified pathogens with 94% accuracy (76% sensitivity and 100% specificity). While use of this system demonstrated remarkably low false-positive rates at only one out of the 36 samples tested (a high specificity), the system remains somewhat limited in sensitivity as indicated by the false negatives. Generally, the platform allowed detection of pathogens with Ct values of 32 and more. This finding was consistent with that reported from other studies (Vora *et al.*, 2004 and Lin *et al.*, 2006) where real-time RT-PCR based detections were found to have better sensitivity than microarray platforms. This is, however, not surprising as the use of specific primers of real-time RT-PCR is more sensitive than those of random

amplifications (Wong *et al.*, 2007; Lee *et al.*, 2008). As limitations in sensitivity can result in false negatives, especially if a patient is tested early or late in the infection or the pathogen of interest does not typically replicate to high titer, it is of the utmost importance that sensitivity of this technology be improved before use is approved in clinical microbiology laboratories.

In the present study, microarrays were able to detect HRV virus in patient 639 whilst use of the RT-PCR assay was unable to detect the virus in this patient. This could be due to the fact that the real-time RT-PCR primers and probe used for the detection of HRVs were only capable of identifying approximately 70% (Contoli *et al.*, 2006) of the strains, thus failing to detect the present of HRVs. It was possible that the microarray correctly detected a rhinovirus strain that real-time RT-PCR failed to detect. Similarly, the pneumovirus genus detected in patient 283 could not be verified by real-time RT-PCR, possibly owing to subtle genetic variations that prevented primer and probe annealing. Thus, in this case, the greater genomic coverage offered by the microarray might, in some cases, provide a more sensitive and accurate detection capability than pathogen-specific real-time RT-PCR. In addition, current evidence has shown our results obtained to be more sensitive than the traditional approaches, including antigen-based assays (Henrickson *et al.*, 2007; Wong *et al.*, 2007; Wang *et al.*, 2008). Thus, if a correct sample (nasopharyngeal samples) is to be taken by trained personnel at the correct time (where viral shedding occurs); the array will detect the presence of

potential pathogen/s more frequently than traditional methods and only slightly less frequently than quantitative real-time RT-PCR, as demonstrated in this current study.

The optimised microarray assay used in the present study, correctly detected the presence of co-infecting pathogens in two of the 36 samples screened and correctly identified the presence of human rhinovirus with high accuracy (94%) and sensitivity (76%), demonstrating the unique potential of this platform to reveal complex disease etiologies and thus bringing the microarray technology closer to use in the clinical settings. Through their ability to simultaneously investigate thousands of potential pathogens in order to make a diagnosis, DNA microarrays have the potential to revolutionize clinical diagnostics. In addition, the use of the PDA algorithm designed for this study also potentially allowed the detection of novel pathogens which will be important in the areas of pathogen discoveries, though more trials need to be conducted to establish this aspect. However, questions remain regarding their sensitivity and reliability. Future improvements will include significant reductions in microarray manufacturing and usage costs. Multiplex microarray formats and “re-usable” arrays are developing technologies that promise to drive down these costs. Furthermore, alternative technologies, such as beads, microfluidics, and nanotube microarrays, might provide advantages in both assay cost and speed relative to traditional microarray platforms. Technologies considerations aside, the advantages of a highly

parallel, nucleic-acid based screening approach for detecting disease pathogens are clear. Validations in larger patient cohorts and in diverse clinical settings will be an important next step towards establishing the clinical role of pathogen detection microarrays.

CHAPTER 4 – PREVALENCE OF RSV, HMPV AND HRV IN COMMUNITY ACQUIRED PNEUMONIA IN A PAEDIATRIC COHORT

4.1 Introduction

Childhood community-acquired pneumonia is a common and serious illness and a leading cause of death especially in developing countries accounting for up to 5 million deaths annually among children younger than 5 years old (Sinaniotis, 2004; Atkinson *et al.*, 2007). In Australia and New Zealand, 23.9% of all paediatric hospital admissions were due to lower respiratory infections and this accounted for the largest group of paediatric admissions (Duncan *et al.*, 2005). Pneumonia, the most severe form of acute lower respiratory tract infections, is up to 10 times higher in developing countries than in developed countries and tends to occur more often early in life (Sinanioti, 2004; Chiang *et al.*, 2007). It is diagnosed in approximately 20 of 1000 infants younger than 1 year of age, in 40 of 1000 children aged 1 to 5 years and in 7 per 1000 of adolescents (Sinaniotis , 2004). More recent studies conducted in Australian children between 5 – 14 years with pneumonia, reported an estimate incidence of 7.6/1000 person-years from non-Indigenous population (Atkinson *et al.*, 2007) and a 5.2/1000 person-years in non-Indigenous children of the same age group (Burgner *et al.*, 2005).

Determining the causative organism of pneumonia in children is challenging and is usually only attempted in cases severe enough to require hospital admission of the affected child (Sinaniotis, 2004). Children are generally not sputum-producers and less than 10% of pneumonia is associated with

bacteraemia and serological responses are poor in patients under one years of age (Sinaniotis, 2004l; Cham *et al.*, 2009). Samples aspirated from the nasopharynx provide the chance of identifying viral etiology by virus isolation, detection of virus antigen or by using PCR techniques (Mahony, 2008). There have been many studies (Bellau-Pujol *et al.,* 2005; Brittain-Long *et al.*, 2008; Mahony, 2008) from various countries attempting to determine local pathogens responsible for community-acquired pneumonia (CAP) and some of these studies indicate that 20% – 60% of confirmed pneumonia do not have an identifiable pathogen (Michelow *et al.*, 2004; Farha *et al.,* 2005) . Nevertheless, with the advances in detection methods for viruses, a number of studies have shown that viruses actually out-number bacteria as causes of childhood CAP, especially in children under 2 years of age (Table 4.1) (Sinaniotis, 2004; Michelow *et al.,* 2004; Farha *et al.,* 2005).

Respiratory syncytial virus (RSV) has been found to be one of the most common viral pathogens in infancy (Nokes *et al.*, 2008; Oliveira *et al*., 2008). However, children with RSV infections are also exposed to a variety of other viruses with similar seasonal patterns, mainly during the winter months, such as influenza, parainfluenza, rhinoviruses (HRV) and human metapneumovirus (hMPV) (Calvo *et al.*, 2008). Thus, to determine both the prevalence of multiple viral respiratory infections in children with ALRI and the effect of the detection of multiple viruses on the severity of diseases, a molecular

Table 4.1. Etiologies of childhood community-acquired pneumonia.

References	Number of patients	Viral (%)	Bacterial (%)	Mixed (%)	No organism identified (%)
Michelow *et al.*, (2004)	154	55	73	28	32
Turner *et al.*, (1987)	98	39	19	10	52
Claesson *et al.*, (1989)	336	25	19	4	52
Nohynek *et al.*, (1991)	121	25	25	20	30
Ruuskanen *et al.*,(1992)	50	26	28	34	12
Gendrel *et al.*, (1997)	104	21	47	8	16
Heiskanen-Kosma *et al.*, (1998)	201	15	41	10	44
Wubbel *et al.*, (1999)	168	17	25	23	57
Juven *et al.*, (2000)	254	32	22	30	16

Note: Due to the present of mixed infections, percentages of studies will not add up to 100%. Adapted from Sinaniotis *et al.*, 2004 and Farha *et al*, 2005.

approach using specific real-time reverse transcription (RT) – PCR assay was adopted to detect for the presence of RSV, hMPV and HRVs in 352 nasopharyngeal samples. RSV was chosen as the first virus to investigate as it is the most common pathogen causing ALRI in children and infants, while hMPV was chosen as the next virus to screen for as it has clinical symptoms almost indistinguishable from RSV. Lastly, HRVs was included in the study as initial microarray testing detected HRVs in nine out of 36 samples screened.

The use of more sensitive and rapid molecular methods from recent developments in the clinical microbiology field had resulted in more studies reporting the presence of mixed viruses in the same clinical specimens, signifying the possibility of multiple infections especially in the case of respiratory diseases (Kaida *et al.*, 2007; Manoha *et al.*, 2007). The incidence of dual respiratory viral infections varies from 10% to 30% in hospitalized infants particularly with respiratory syncytial virus (RSV) and human metapneumovirus (hMPV) and RSV and human rhinoviruses (HRVs) (Richard *et al.,* 2008). It may be assumed that the simultaneous presence of more than one viral pathogen would be associated with epidemiologic and clinical features that differ from single infections, especially with respect to disease type and severity. However, controversial reports have emerged suggesting either an association between dual infections and an increase in the disease severity or the absence of an association between dual infections

and an increase in the disease severity (Paranhos-Baccala *et al.*, 2008). Thus, to test the hypothesis that dual infections do lead to more severe diseases, we compared viruses detected in our clinical samples and correlated these with patients' clinical symptoms, which were defined as severe pneumonia and mild pneumonia (previously defined in Chapter 2.1).

In the temperate regions of the world, a clear seasonal variation in the occurrence of respiratory tract infections, a large proportion of them which are viral, peaks during the cold winter months (Welliver *et al.*, 2007; Yusuf *et al.*, 2007). This variation is less apparent in the tropics, where there is less fluctuation than that reported for cases recorded in environments of ambient temperature (Shek *et al.*, 2003). Nonetheless, there is evidence that the incidence of some of the respiratory viruses, in particular respiratory syncytial virus (RSV), shows seasonal trends and varies with prevailing environmental conditions (Shek *et al.*, 2003; Lee *et al.*, 2007; Yusuf *et al.*, 2007; Omer *et al.*, 2008).

In the tropical areas of the world, the average temperature is higher and the seasonal change in temperature is less than that recorded in temperate regions of the world. However, these latter regions do experience local variations in temperature, humidity and rainfall (Shek *et al.*, 2003; Welliver *et al.*, 2007; Omer *et al.*, 2008). Even though countries in the tropics are buffered from the harsh conditions of the winter, the toll of respiratory infections is still high in

these areas of the world. In Singapore, they account for up to 50% of the pediatric outpatient cases seen annually (Shek *et al.*, 2003; Chiang *et al.*, 2007). In Malaysia, they were the fourth leading cause for hospital admissions in children under the age of 5 years (Shek *et al.*, 2003). With the development of vaccines and anti-virals for prophylaxis and treatment of respiratory virus infections, an awareness of the epidemiology and seasonality of these infections is needed for optimizing healthcare strategies in the tropics.

In general, our knowledge of how epidemics of respiratory viruses are initiated and sustained is incomplete. For RSV, it was noted that geographical regions, mainly the Northern and Southern hemispheres with temperate climates, has epidemic peaks during winter (Panozzo *et al.*, 2007; Zlateva *et al.*, 2007). This is in contrast to countries located near the equator (Singapore and Malaysia) where temperatures are very constant and rainfall is generally heavy and RSV activity was noted continuously throughout the year (Shek *et al.*, 2003). Peak activity of RSV occurred from March to August in Singapore, whilst no clear seasonal predominance was observed in Malaysia (Shek *et al.*, 2003; Welliver, 2003; Yusuf *et al.*, 2007). Moving slightly northward where climates are hot in West Africa and India, RSV activity peaks in late summer and early autumn in each area (Cherian *et al.*, 1990; Weber *et al.*, 1998; Yusuf *et al.*, 2007). RSV activity in sub-tropical countries, for example Mexico City, Hong Kong and Dhaka (Bangladesh) where climates are cooler than those of tropical countries, noted that the presence of RSV is continual i.e. throughout

the year; peaks , however, do occur in late summer and early autumn and winter peaks also appears in Dhaka (Sung *et al.*, 1992; Mullins *et al.*, 2003). Generally, it has been suggested that cold weather may increase RSV activity, however it was noted that RSV activity was described as being continuous throughout the year in warm equatorial areas, suggesting that temperature cannot be the only factor influencing the activity of the virus. Thus, we undertook the present study in an attempt to gain a better understanding of the respiratory viral activities in Indonesia and to try to determine the local environmental conditions that might have also played a role in the incidences of infections caused by these pathogens.

4.2 Materials and Methods

4.2.1 Detection Of RSV, hMPV And HRV Viruses Using Reverse Transcriptase RT-PCR

A total of 352 nasopharyngeal samples from 256 patients enrolled in the Indonesian paediatric cohort study were used for this study. All samples collected were aliquot into 384 wells PCR plates and subjected to real-time RT-PCR detections of RSV A, RSV B, hMPV, HRV and 18S rRNA. Briefly, a 20 µL reaction mixture containing 2 µL of the purified patient RNA, 5 U of MuLV reverse transcriptase, 8 U of recombinant RNase inhibitor, 10 µL of 2X universal PCR Master Mix with no UNG (all from Applied Biosystems, Foster City, CA, USA) was combined with 0.9 µM primer and 0.2 µM (RSV B, hMPV and 18S rRNA), 0.3 µM (HRV) or 0.5 µM (RSV A) probe. The primers and probe sequences for hMPV were: 5'-AGCAAAGCAGAAAGTTTA TTCGTTAA-3'; 5'-ACCCCCCACCTCAGCATT-3'; and 5'-FAM-ATTCATGCAA GCTTATGGTGCTGGTCAAA-TAMRA-3'. Primers and probes for RSV (Hu *et al.,* 2003), HRV (Contoli *et al.,* 2006) and 18S rRNA (Muthukumar *et al.,* 2005) have been previously described. Prior to the start of real-time RT-PCT assays, samples were aliquot into 96 wells format for usage.

Samples underwent reverse transcription at 48°C for 30 minutes, then were heated at 95°C for 10 minutes and amplified by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C on an ABI Prism 7900HT Sequence Detection System (Applied Biosystems). During amplification, fluorescence emissions were monitored at every thermal cycle. The threshold (Ct) represents the cycle at which significant fluorescence is first detected. Ct value was converted to copy number using a control plasmid of known concentration (calculated with the use of spectrophotometer: RSV A, 1.1×10^9 copies/ µL had a Ct value of 14.3; RSV B, 3.1×10^9 copies/ µL had a Ct value of 10.2; hMPV, 7.5×10^9 copies/ µL had a Ct value of 10.5; HRV, 1.7×10^7 copies/ µL had a Ct value of 20.2; 18S rRNA, 3.9×10^9 plasmid copies/ µL had a Ct value of 10.5. Negative controls and serial dilutions of a plasmid clone (positive control) were included in every real-time RT-PCR assay and all runs were done in duplicates. Assay for 18S rRNA was run in conjunction with every single viral assay by using the same samples aliquot into the 96-wells PCR plates. Viral copy numbers were normalized to human cells collected in the nasal wash using the 18S rRNA copy number in each sample. We estimated the number of cells present in each sample from our measurement of an average of 1.11×10^7 18S rRNA copies per cell, using primers, probe and a standard curve as previously described (Muthukumar *et al.*, 2005). Thus, viral copy numbers per cell in the nasal wash samples were obtained using the formulae: (viral copy number/µL) / (number of cells/µL).

4.2.2 Standardisation of Nasal Wash Samples

Similar to other *Paramyxoviridae*, RSV and hMPV particles are mostly cell-associated in infected tissues (Gueudin *et al.,* 2003) and as nasal aspirates are non-homogenous, a method for standardisation of samples based on quantitation of the mRNA of a housekeeping gene, 18S rRNA was used. An extensive literature survey resulted in the finding that 18S rRNA is expressed at a constant rate in many cells including T-lymphocytes and was therefore good for the purpose of normalisation of nasopharyngeal cells in the present study (Casabianca *et al.*, 2004; Bas *et al.*, 2004; Morse *et al.*, 2005). Using the 18S rRNA, the amount of human material in each sample could be evaluated, and an estimate made of the number of human cells contained in each sample evaluated in this study. Thus, real-time RT-PCR used in this study was designed to quantify viral genomic RNA and human 18S rRNA. These methods then were used to assess the viral loads in respiratory specimens from children for correlation with disease severities. Primers and probe for the detection of 18S rRNA were adopted from Muthukumar *et al.*, (2005).

4.2.3 Weather Correlation Analysis.

Correlations between weather parameters (maximum weekly temperature, minimum weekly temperature, average weekly humidity and average weekly rainfall) and (1) RSV, (2) HRV, (3) hMPV and (4) the proportion of severe cases, were quantified using the non-parametric Spearman's rho coefficient for lags up to 5 weeks. To account for possible autocorrelation, first-order differencing was performed on disease incidence variables. The proportion of severe cases was computed as the number of severe cases out of all reported cases with lower respiratory infection.

4.3 Results

4.3.1 Assays Development

The strategy used for this research was to employ a novel microarray chip technology for the screening of possible viruses present in nasopharyngeal samples collected from an Indonesian birth cohort. To do so, it was necessary to first evaluate the use of the newly developed microarray platform and then compare it with a highly sensitive and reproducible detection method, namely Taqman-based real-time RT-PCR assays. Primers and probes used for the real-time RT-PCR method were either developed in the present study or tested and optimised using those previously published. Respiratory syncytial virus (RSV) was first chosen as it was the single most important etiological agent in infancy and is a major cause of bronchiolitis and pneumonia in children under 2 years of age (Mahony, 2008). hMPV was chosen next as the clinical manifestations of its' infection in young children are indistinguishable from the clinical manifestations of RSV infection (Kahn, 2006). In addition, there are many studies that demonstrated hMPV is responsible for a substantial proportion of lower respiratory tract infections in children and is second only to RSV as a cause of bronchiolitis in early childhood (Boivin *et al.*, 2003; Chano *et al.*, 2005; Kahn, 2006). As mentioned in Chapter 3.3 (page 91),

primers and probe used for HRV detection was optimised for use by Contoli *et al.*, (2006), thus no assay development was performed for this study.

4.3.1.1 Human Metapneumovirus

To detect hMPV in the 352 nasopharyngeal samples using real-time RT-PCR, many published primers and probes were chosen and tested with the reference strains CAN98-75 and CAN97-83 (Bastien *et al.*, 2004; van den Hoogen *et al.*, 2004; Huck *et al.*, 2006). However, limited success was obtained following this protocol and many primers and probe sets did not generate any readings on the two reference strains, even after multiple attempts to optimise the PCR conditions. Thus, the decision was taken to design new primers and probes in the present investigation and use these to detect the presence of hMPV in our nasopharyngeal samples.

Firstly, known hMPV nucleotide sequences deposited in Genbank, NCBI were extracted and aligned with Clustal W software and highly conserved genomic regions, namely nucleoprotein gene and fusion protein gene were chosen to use as subsets for primers and probes design. Next, using Primer Express version 1.0 software (Applied Biosystems, USA), a total of ten primer sequences for forward primers, reverse primers and probe sequences

Table 4.2. Primers and probes designed and trialled for Taqman amplification of viral RNA from hMPV.

Primer/ Probe	Sequences: (5' – 3')	Targeted region	Sequence Type	Size (bp)
F1FT	GCTGTTCCATTGGCAGCAA	Fusion protein	Forward primer	19
F1RT	TCTGCATCTTGGTTGGTTATATA	Fusion protein	Reverse primer	23
F2RT	TCTGCGTCTTGGTTGGTTATATA	Fusion protein	Reverse primer	23
F1PT	FAM-TAGGGATCATCAAGCAGCTGAACAAAGGTTG-TAMRA	Fusion protein	Probe	31
F2PT	HEX-TAGGGATCATCAAGCAGCTGAACAAGGGTTG-TAMRA	Fusion protein	Probe	31
N1FT	AGCAAAGCAGAAAGTTTATTCGTTAA	Nucleo-protein	Forward primer	26
N2FT	AGCAAAGCACAAAGTTTATTCGTTAA	Nucleo-protein	Forward primer	26
N1RT	ACCCCCCACCTCAGCATT	Nucleo-protein	Reverse primer	18
N1PT	FAM-ATTCATGCAAGCTTATGGTGCTGGTCAAA-TAMRA	Nucleo-protein	Probe	29
N2PT	HEX-ATTCATGCAAGCTTACGGTGCTGGTCAAA-TAMRA	Nucleo-protein	Probe	29

were designed with regard to general rules of primer and probe design (Table 4.2). To determine the specificity of these primers and probes to hMPV, they were blasted in NCBI to ensure that they do not cross align with genomes from other viruses, bacteria or *Homo sapiens*. Before commencing on the laboratory experiments, primers and probe sets were input into the Oligo6 Primer Analysis software for the designing of optimal primers and probe conditions. The fluorogenic probes chosen for use in detection were either 5' reporter dye 6-carbosy-fluorescein (FAM) or 5' reporter dye hexachloro-6-carboxy-fluorescein (HEX) and the 3' quencher dye 6-carboxy-tetramethyl-rhodamine (TAMRA).

During the experiments, different combination of primers and probes were trialled to determine sensitivity and specificity of the sets. In addition, different probe concentrations of 200nM, 300nM, 400nM, 500nM and 900 nM were also trialled with ten-fold dilutions of CAN98-75 and CAN97-83 clones. Eventually, hMPV_N1FT, hMPV_N1RT and hMPV_N1PT labelled with 5'FAM and 3'TAMRA at 200nm, detecting for nucleoprotein gene of hMPV were chosen for their sensitivity (as it was able to detect controls), specificity (with no unspecific bindings) and reproducibility (Appendix 5).

4.3.1.2 Respiratory Syncytial Virus

To detect for RSV (i.e. both RSV A and RSV B), primers and probes based on relevant information from published literatures (van Elden *et al.,* 2003; Hu *et al.,* 2003 and Kuypers *et al.,* 2004) were evaluated in the present study. Primers and probe sets from Hu and colleagues (2003) were selected as they allowed the detection of the RSV A and B viral clones up to 10^6 dilutions of the original concentrations. Target regions for amplification were the conserved portion from the nucleoprotein genes of both RSV A and RSV B.

4.3.2 Viral Etiology in Community Acquired Pneumonia

A total of 352 nasopharyngeal samples from 256 patients enrolled in the Indonesian paediatric cohort were screened for the presence of RSV, hMPV and HRVs. One hundred and eighty-seven or 73% of patients had one episode of either mild or severe pneumonia; 52 (20.3%) of the patients had two episodes of (either mild or severe) pneumonia; 11 (4.3%) had three episodes of either mild or severe); four (1.6%) had four episodes of (either mild or severe) pneumonia; one (0.4%) had five episodes and another had six episodes of pneumonia in the 28 months of sample collections (Figure 4.1).

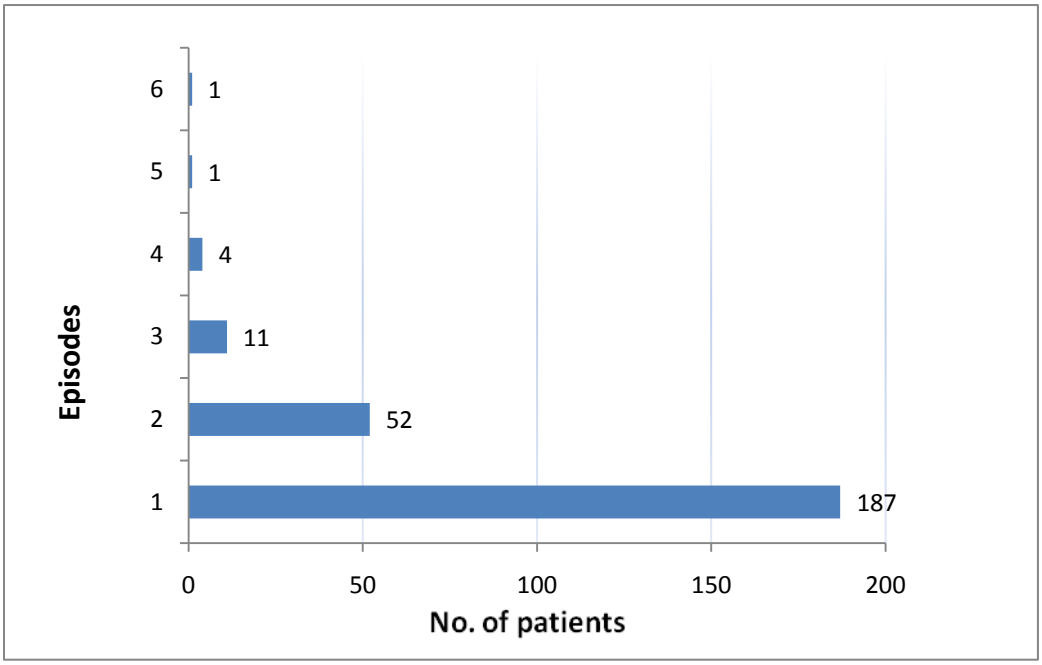


Figure 4.1. Number of episodes of pneumonia infections in an Indonesian paediatric cohort. A total of 256 patients were enrolled for this study. One hundred and eighty-seven patients had one episode of pneumonia, 52 had 2 episodes, 11 had 3 episodes, 4 had 4 episodes, 1 had 5 and the other had 6 episodes of pneumonia in the 28 months of sample collections.

As represented in Figure 4.2, RSV A, RSV B, hMPV and HRV were not detected in 151 (42.9%) nasopharyngeal samples, while the remaining 201 (57.1%) had either one or more viruses detected. In all, HRVs were detected in a total of 119 (33.8%) out of the 352 nasopharyngeal samples screened. In 85 (24.1%) samples, RSV (genotypes A and B) were less prevalent than HRV while hMPV accounted for approximately 8.5% (30 episodes) of all pneumonia screened (Figure 4.3). Of the 201 episodes, 31 episodes (15.4%) had more than one virus detected.

Two hundred and eighty (79.5%) of the samples were collected from patients suffering from mild episodes (tachypnoea with no chest indrawing) of pneumonia. As there were only three cases of very severe pneumonia, which is statistically too small for analysis, these cases were incorporated into the 69 cases of severe pneumonia to strengthen the statistical analysis of this study. Thus, 72 (20.5%) of these episodes were from patients suffering from both severe (lower chest wall indrawing with or without tachypnoea) and very severe (either lethargy, difficulty to arouse, or general danger signs) pneumonia. For mild pneumonia, HRV was detected in 91 (32.5%), RSV A in 53 (18.9%), hMPV in 24 (8.6%) and RSV B in 11 (3.9%) of the episodes (Figure 4.2). In severe pneumonia, HRV accounted for 28 (38.9%), RSV A for 20 (27.8%), hMPV for 6 (8.3%) and RSV B for 1 (1.4%) of the episodes. In one hundred and twenty-five (44.6%) episodes of mild pneumonia and 26

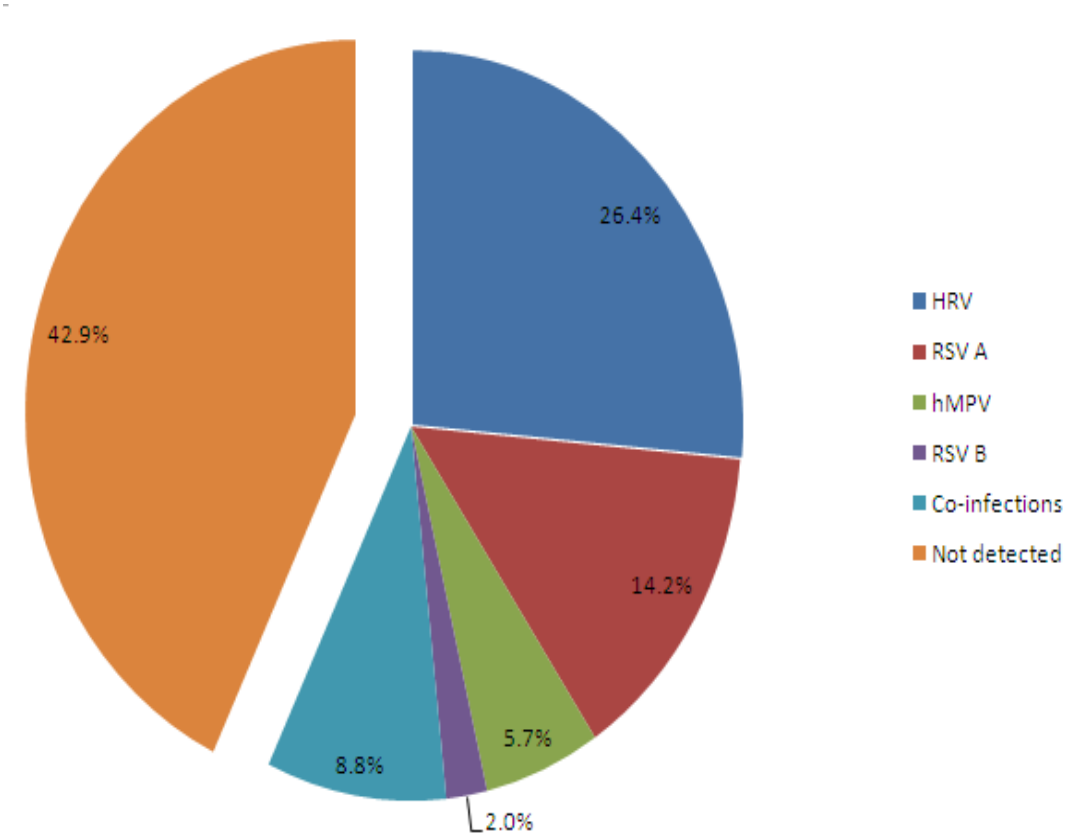


Figure 4.2 Distribution of viruses detected in nasal washes from 352 episodes of ALRTIs from an Indonesian paediatric cohort. HRV was detected in 93 (26.4%) episodes of pneumonia; RSV A in 50 (14.2%) episodes, hMPV in 20 (5.7%) episodes, RSV B in 7 (2%) of episodes of pneumonia. Thirty-one (8.8%) episodes had co-infections of either 2 or more viruses and 151 (42.9%) episodes had neither HRV, RSV or hMPV detected.

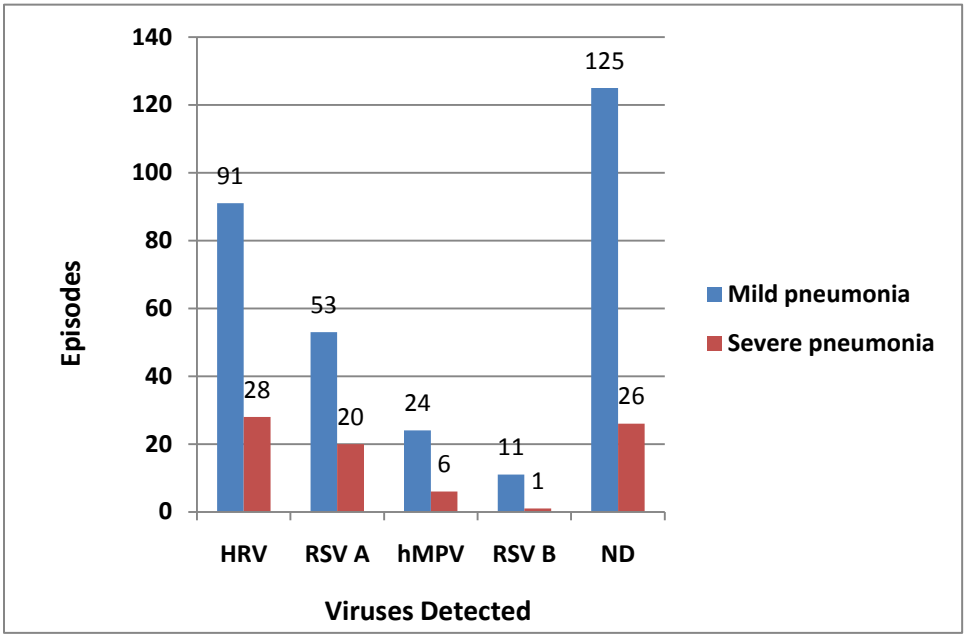


Figure 4.3. Viruses detected in nasopharyngeal samples of patients showing mild or severe pneumonia.
HRV: human rhinoviruses, RSV A: respiratory syncytial virus A, hMPV: human metapneumovirus, RSV B: respiratory syncytial virus B, ND: none detected.

(36.1%) of severe pneumonia; HRV, RSV A, hMPV and RSV B could not be detected (Figure 4.3).

In all, HRV was the most common respiratory virus detected in this cohort of patients suffering from pneumonia. To determine the role of HRV and it association with subsequent respiratory viral infections, a breakdown of the clinical features of six patients with more than four episodes of pneumonia was tabulated (Table 4.3). All six patients had at least one episode of infection caused by HRV, though they tended to have mild pneumonia, especially when HRV was the only causative agent. Notably, three other patients with subsequent hMPV or RSV A viral infections tended to have severe pneumonia, except for patient 1150110.

From this study, the four pathogens (RSV A, RSV B, hMPV and HRV) investigated were detected more commonly in severe pneumonia (63.9%) than in mild pneumonia (55.3%). HRV was detected in 32.5% of all mild pneumonia versus 38.9% of all severe pneumonia. RSV A was detected in 18.9% of mild versus 27.9% of severe pneumonia. hMPV was detected in 8.6% of mild versus 8.3% of severe pneumonia and RSV B was detected in 3.9% of mild versus 1.4% of severe pneumonia. One hundred and twenty-five of the episodes (44.6%) in mild pneumonia and 26 of the episodes (36.1%) in severe pneumonia did not have RSV A, RSV B, hMPV nor HRV present.

Table 4.3. Clinical features of children with more than 4 episodes of pneumonia infections.

				Viruses Detected				
Patient No	**DOB**	**CD**	**Pneumonia**	**RSV A**	**RSV B**	**hMPV**	**HRV**	**ND**
1150110	**05/05/98**	**26/11/99**	**Mild**			+		
		5/7/00	**Mild**					+
		12/1/01	**Mild**				+	
		23/4/01	**Mild**	+		+		
1150312	**3/2/98**	**19/4/99**	**Mild**	+				
		22/5/00	**Mild**					+
		31/10/00	**Mild**					+
		4/4/01	**Mild**		+		+	
1260506	**29/11/99**	**6/6/00**	**Mild**				+	
		3/7/00	**Mild**					+
		24/2/01	**Severe**	+			+	
		30/4/01	**Severe**	+				
1280105	**7/5/99**	**20/1/00**	**Mild**				+	
		28/10/00	**Mild**					+
		21/11/00	**Mild**				+	
		7/3/01	**Mild**				+	
1250504	**22/2/98**	**30/9/99**	**Mild**					+
		20/10/99	**Mild**					+
		4/5/00	**Mild**		+		+	
		12/8/00	**Severe**	+				
		13/3/01	**Mild**				+	
1220511	**4/1/99**	**22/3/00**	**Mild**	+				
		1/5/00	**Mild**					+
		19/5/00	**Mild**					+
		5/10/00	**Mild**				+	
		18/1/01	**Severe**			+		
		28/4/01	**Severe**			+		

DOB: date of birth, CD: sample collection date, HRV: human rhinoviruses, RSV A: respiratory syncytial virus A, hMPV: human metapneumovirus, RSV B: respiratory syncytial virus B, ND: none detected.

Risks in developing severe pneumonia were similar for RSV A (27.1%), HRV (23.5%) and hMPV (20.0%).

4.3.3 Co-infections and Correlation With Disease Severity

Of the 352 nasopharyngeal samples collected, 31 episodes had more than one virus detected, accounting for 8.8% of all samples tested or 15.4% of the 201 co-infections caused mild pneumonia while 9 or 29% caused severe pneumonia. Figure 4.4 shows a breakdown of mild and severe pneumonia according to the viruses detected in the samples used in this study: i.e. HRV, RSV A, hMPV and RSVB viruses that were detected. The bar chart further stratifies the figures into those infections caused by either single or multiple virus infections. For HRV, co-infections for mild pneumonia accounted for 17 out of 92 episodes (18.5%) while co-infections for severe pneumonia accounted for 9 out of 27 episodes (33.3%). For RSV A, co-infections for mild pneumonia and severe pneumonia accounted for 15 out of 53 episodes (28.3%) and 8 out of 20 episodes (40.0%), respectively. For hMPV, co-infections in mild pneumonia stood at 37.5% and for severe pneumonia was 16.7%. Co-infections for RSV B were only noted in mild pneumonia (45.5%).

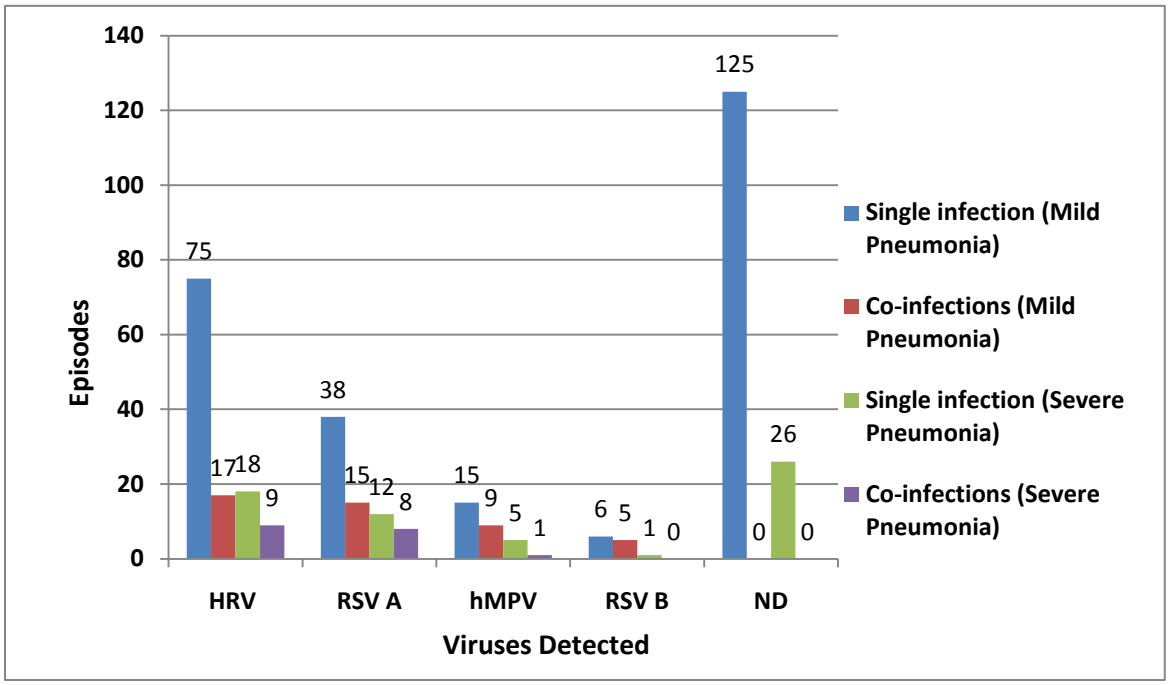


Figure 4.4 Total number of ALRTIs episodes categorized by infecting virus or viruses. A breakdown of mild and severe pneumonia according to the viruses detected in the samples used in this study is as shown. The bar chart further stratifies the figures into those infections caused by either single or multiple virus infections. HRV: human rhinoviruses, RSV A: respiratory syncytial virus A, hMPV: human metapneumovirus, RSV B: respiratory syncytial virus B, ND: none detected.

Dual infections detected in the 31 episodes of mild and severe pneumonia were combinations of both the (a) HRV and RSV A viruses (b) HRV and RSV B viruses (c) HRV and hMPV viruses and (d) RSV A and hMPV viruses. In two episodes of mild pneumonia, mixed infections with HRV, RSV A and hMPV viruses were detected (Table 4.4). To determine if these co-infections were significantly associated with severe pneumonia, *P* values were calculated. Using two-tailed Fisher's exact test, statistical analyses were performed for all co-infections relative to the number of episodes of mild pneumonia (72 episodes) and severe pneumonia (280 episodes). Significance of $P = 0.006$ was obtained for HRV and RSV A virus infections whilst no significant differences (Table 4.4) were observed for all other co-infections. Relative risks (RR) were also calculated (relative risk is calculated to determine the risk of developing severe pneumonia due to these co-infections) for HRV + RSV A and HRV + hMPV dual infections as severe pneumonia were noted for these combinations. It was observed that dual infections of HRV + RSV A increased the relative risk of having a severe pneumonia by 4.3 times as opposed to dual infections of HRV + hMPV by only 2.0 times (statistically not significant).

Table 4.4. Disease Severity in Relation to Co-infections.

Types of Co-infections	Disease severity	No. of episodes	Risk of Infection
HRV + RSV A	***Mild***	**8**	***P = 0.006*** **(RR = 4.3)** **(95% confidence: 1.4 to 10)**
	Severe	**8**	
HRV + RSV B	***Mild***	**5**	***P = 0.59***
	Severe	**0**	
HRV + hMPV	***Mild***	**2**	***P = 0.49*** **(RR = 2.0)**
	Severe	**1**	
RSV A + hMPV	***Mild***	**5**	***P = 0.59***
	Severe	**0**	
HRV+RSVA+hMPV	***Mild***	**2**	***P = 1.0***
	Severe	**0**	

Using two-tailed Fisher's exact test, statistical analyses were performed for all co-infections relative to the number of episodes of mild and severe pneumonia. Significance of P = 0.006 was obtained for HRV and RSV A virus infections with a 4.3 times relative risk of developing severe pneumonia due to this combination of co-infections. HRV: human rhinoviruses, RSV A: respiratory syncytial virus A, hMPV: human metapneumovirus, RSV B: respiratory syncytial virus B, Mild: mild pneumonia, Severe: severe pneumonia, RR: relative risk.

4.3.4 Viral Loads and Correlation with Disease Severity

Determination of the viral copy number of RSV A, RSV B, hMPV and HRV in positive samples was done with serial dilutions of a plasmid clone. Known concentrations of plasmid clones for each individual virus were used to construct standard curves for all three viruses (RSV A and B, hMPV and HRV). Figure 4.5 is an example of human metapneumovirus quantitative real-time RT-PCR amplification plot of standard plasmid DNA performed in duplicates using ten-fold dilutions of the clone. For absolute quantification of viral genomes a standard curve was constructed from threshold cycle value viral genomes a standard curve was constructed from threshold cycle value Figure 4.6. A highly significant linear relationship between the log of the input target DNA copy numbers and the Ct values can be observed using linear regression showing R-squared value close to 1. Thus, interpolations by inputting rhinovirus concentration (viral load) of samples containing unknown quantities of rhinovirus-RNA were performed to determine viral copy number of each virus in each of the clinical samples. Standard curves for 18s rRNA (Figure 4.7), RSV A (Figure 4.8), RSV B (Figure 4.9) and human metapneumovirus (Figure 4.10) were also constructed using these formulae.

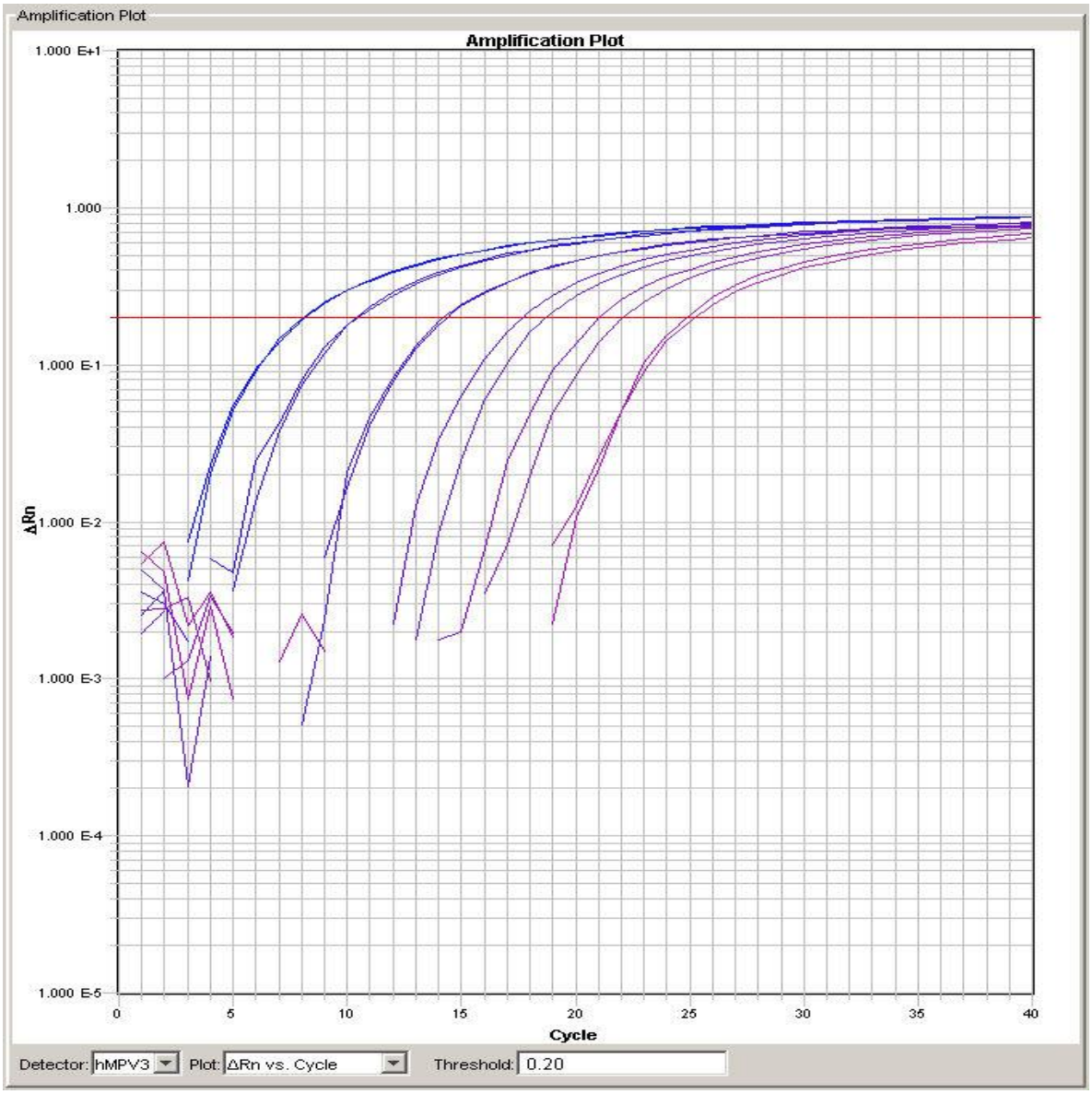


Figure 4.5 Real-time RT-PCR amplification plot for human metapneumovirus plasmid clone. Ten-fold serial dilutions in duplicates were performed to a concentration of 10^6 for the construction of standard curves. All dilutions were performed in duplicate.

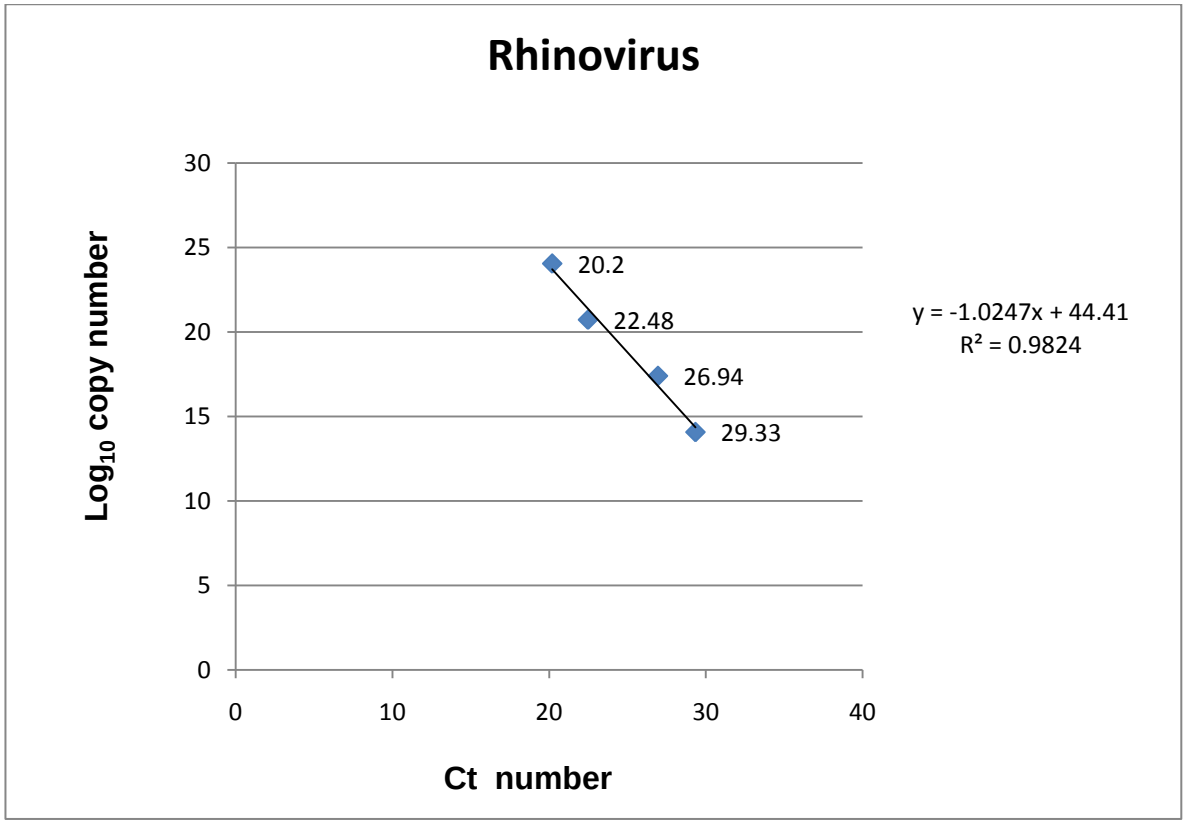


Figure 4.6. A standard curve for rhinovirus plasmid clone constructed using values determined by TaqMan® real-time RT-PCR. Log_{10} copy number is expressed as per µL. All dilutions were performed in duplicate.

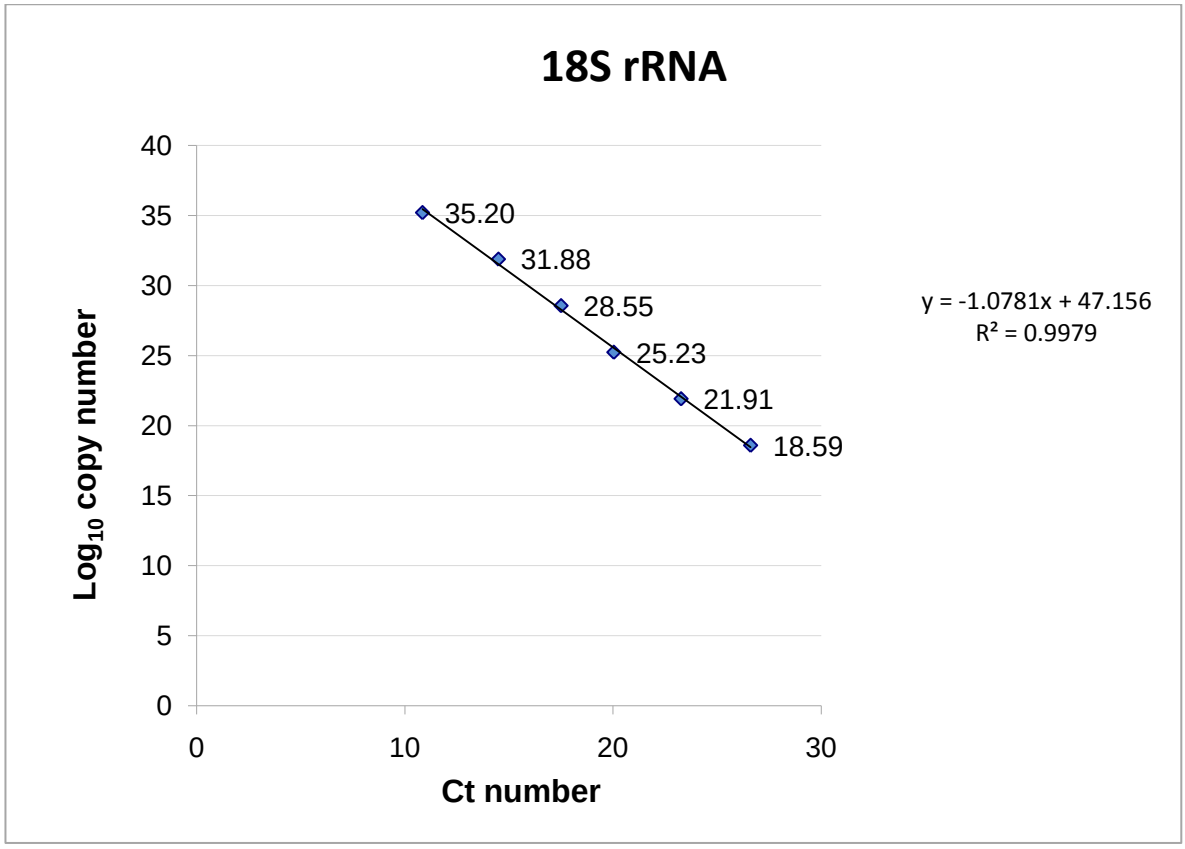


Figure 4.7. A standard curve for 18S rRNA plasmid clone constructed using values determined by TaqMan® real-time RT-PCR. Log_{10} copy number is expressed as per µL. All dilutions were performed in duplicate.

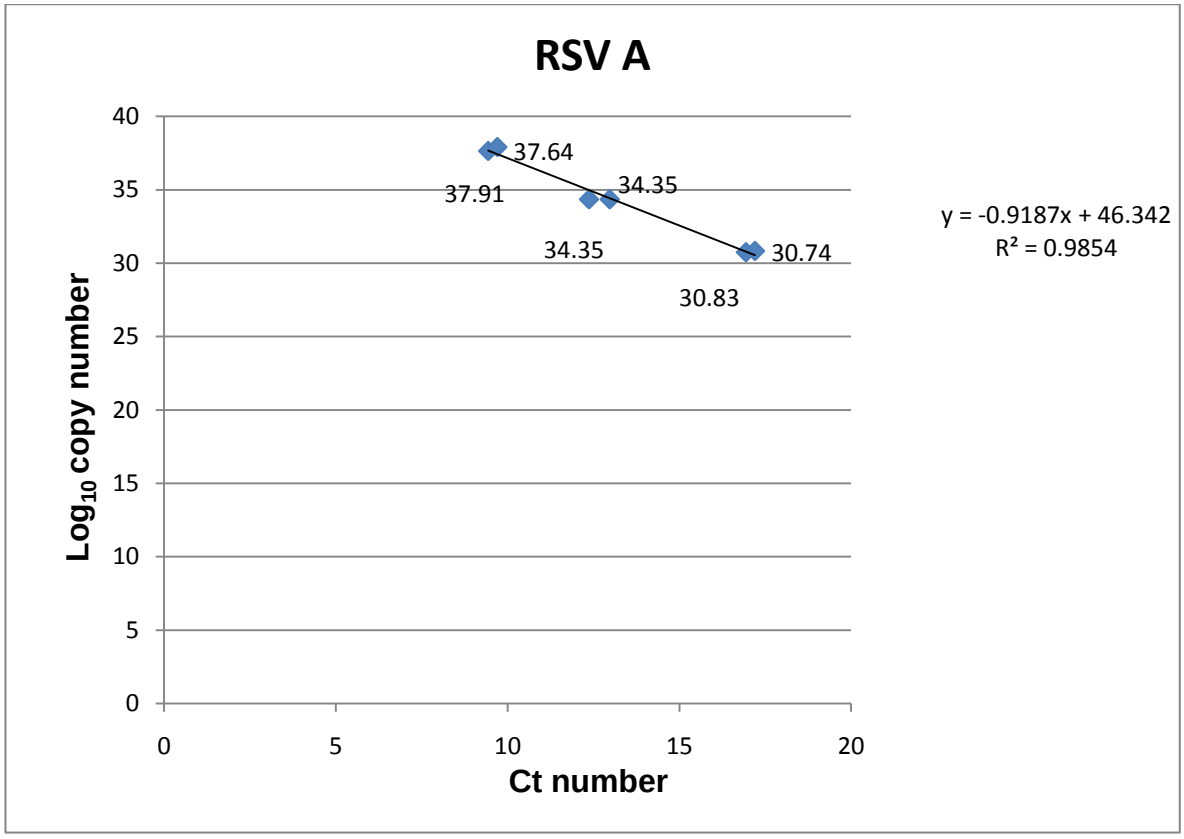


Figure 4.8. A standard curve for RSV A plasmid clone constructed using values determined by TaqMan® real-time RT-PCR. Log_{10} copy number is expressed as per µL. All dilutions were performed in duplicate.

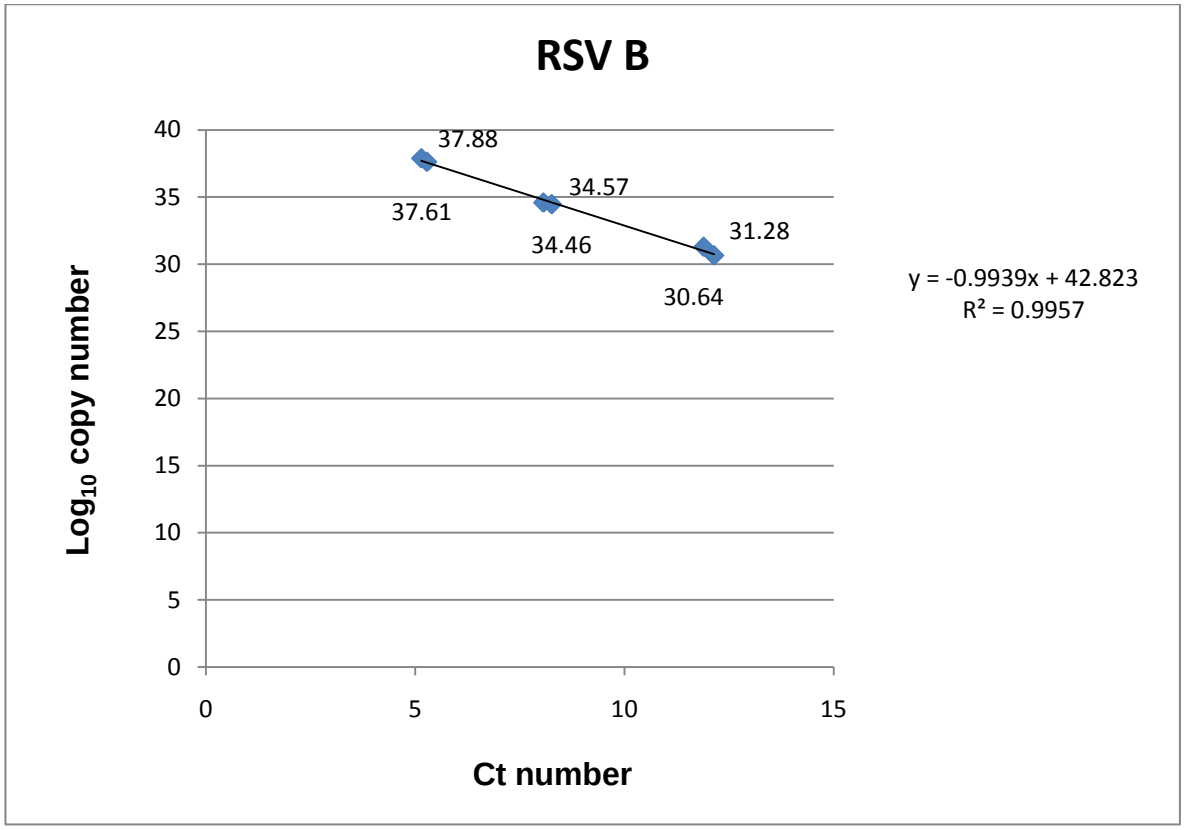


Figure 4.9. A standard curve for RSV B plasmid clone constructed using values determined by TaqMan® real-time RT-PCR. Log_{10} copy number is expressed as per µL. All dilutions were performed in duplicate.

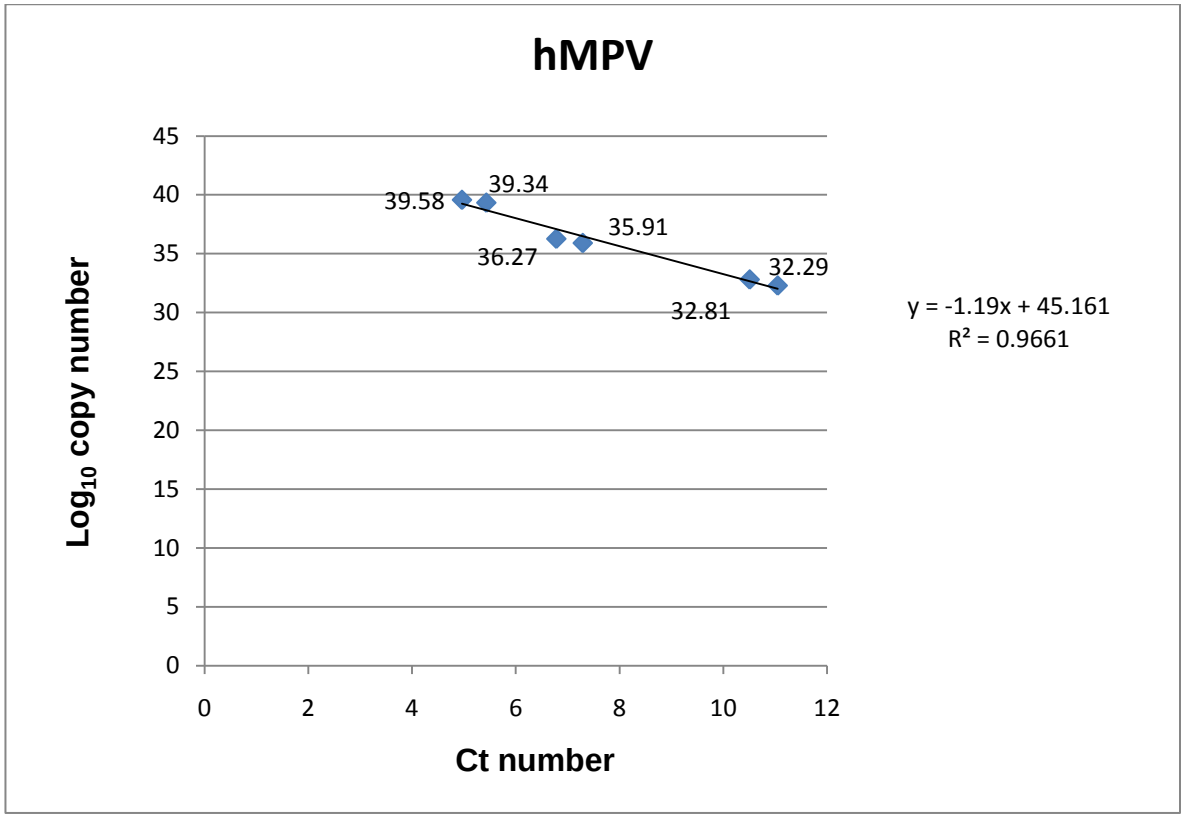


Figure 4.10. A standard curve for hMPV plasmid clone constructed using values determined by TaqMan® real-time RT-PCR. Log_{10} copy number is expressed as per µL. All dilutions were performed in duplicate.

Using the standard curves constructed, viral copy numbers for RSV A in clinical samples ranged from 2.64 X 10^2 to 8.49 X 10^6 per µLof the original sample; for RSV B from 5.47 X 10^4 to 1.30 X 10^8; hMPV from 6.44 X 10^2 to 2.08 X 10^7 and HRV from 3.38 X 10^3 to 1.08 X 10^{10}. The analysis of 18s rRNA copies in each clinical sample confirmed that the number of cells in the nasal aspirates differed from one sample to another, ranging from 5.72 X 10^4 to 5.68 X 10^9. As viral particles are predominantly cell-associated in clinical samples and as the number of copies of the house-keeping gene (18S rRNA) is proportional to the number of cells in the sample, results can thus be normalized to the number of copies of RNA viruses/µL to the number of 18S rRNA copies/µL. This allows the results obtained from different nasal aspirates to be compared with each other, and ensures that the number of cells collected in each nasal sample did not contribute to the viral copy numbers.

To determine if disease severity noted for each sample is associated with viral load of each sample, the median viral copies/µL and median viral copy number/per human cell for all viruses tested, were quantified according to disease severity as shown in Table 4.5. In mild pneumonia, HRV copy number per µLof sample ranged from 3.38 X 10^3 to 1.08 X 10^{10} with a median value of 6.22 X 10^4 and the normalized HRV copy number per human cell ranged from 1.17 X 10^2 to 3.55 X 10^{10} with a median value of 2.32 X 10^4. For severe pneumonia, the HRV number per µL ranged from 5.83 X 10^3 to 2.73 X 10^8 with a median value of 2.01 X 10^5 while the normalized HRV copy

Table 4.5. Disease severity in relation to viral load.

Mild Pneumonia				Severe Pneumonia			
Type of virus detected	**Median Viral copy no./µL**	**Median Cell no./ µL**	**Median Viral copy no./cell**	**Type of virus detected**	**Median Viral copy no./ µL**	**Median Cell no./ µL**	**Median Viral copy no./cell**
HRV n = 91	6.22×10^4	7.43	2.32×10^4	***HRV n = 28***	2.01×10^5	4.59	1.97×10^4
RSV A n = 53	7.40×10^4	4.57	2.76×10^4	***RSV A n = 20***	1.48×10^5	8.38	2.08×10^4
RSV B n = 11	3.57×10^5	4.16	1.55×10^5	***RSV B n = 1***	9.21×10^5	9.73×10^{-1}	9.47×10^5
hMPV n = 24	1.96×10^4	1.03×10^1	3.02×10^3	***hMPV n = 6***	1.93×10^3	5.86×10^{-1}	6.76×10^3
ND n = 125	NA	1.70	NA	***ND n = 26***	NA	3.72	NA

To determine if disease severity for each sample is associated with viral load, median viral copies/µL and median viral copy number/per human cell for all viruses were quantified and compared for both mild and severe pneumonia.
HRV: human rhinoviruses, RSV A: respiratory syncytial virus A, hMPV: human metapneumovirus, RSV B: respiratory syncytial virus B, ND: HRV, RSV A, RSV B and hMPV not detected.

per cell ranged from 6.30 X 10^2 to 7.94 X 10^6 with a median value of 1.97 X 10^4 (Figure 4.11).

For RSV A, viral copy per µL ranged from 2.64 X 10^2 to 8.49 X 10^6 with a median value of 7.40 X 10^4 and normalized viral copies per cell ranged from 5.77 X 10^1 to 2.17 X 10^6 with a median value of 2.76 X 10^4 for mild pneumonia. For severe pneumonia, viral copy per µL ranged from 3.29 X 10^2 to 4.65 X 10^6 with a median value of 1.48 X 10^5 and normalized viral copies per cell ranged from 6.34 X 10^1 to 5.83 X 10^5 with a median value of 2.08 X 10^4 (Figure 4.12).

For RSV B, viral copy per µL ranged from 5.74 X 10^4 to 1.30 X 10^8 with a median value of 3.57 X 10^5 and normalized viral copies per cell ranged from 1.22 X 10^4 to 3.45 X 10^6 with a median value of 1.55 X 10^5 for mild pneumonia. There was only one RSV B positive for severe pneumonia and thus no comparison was done (Figure 4.13).

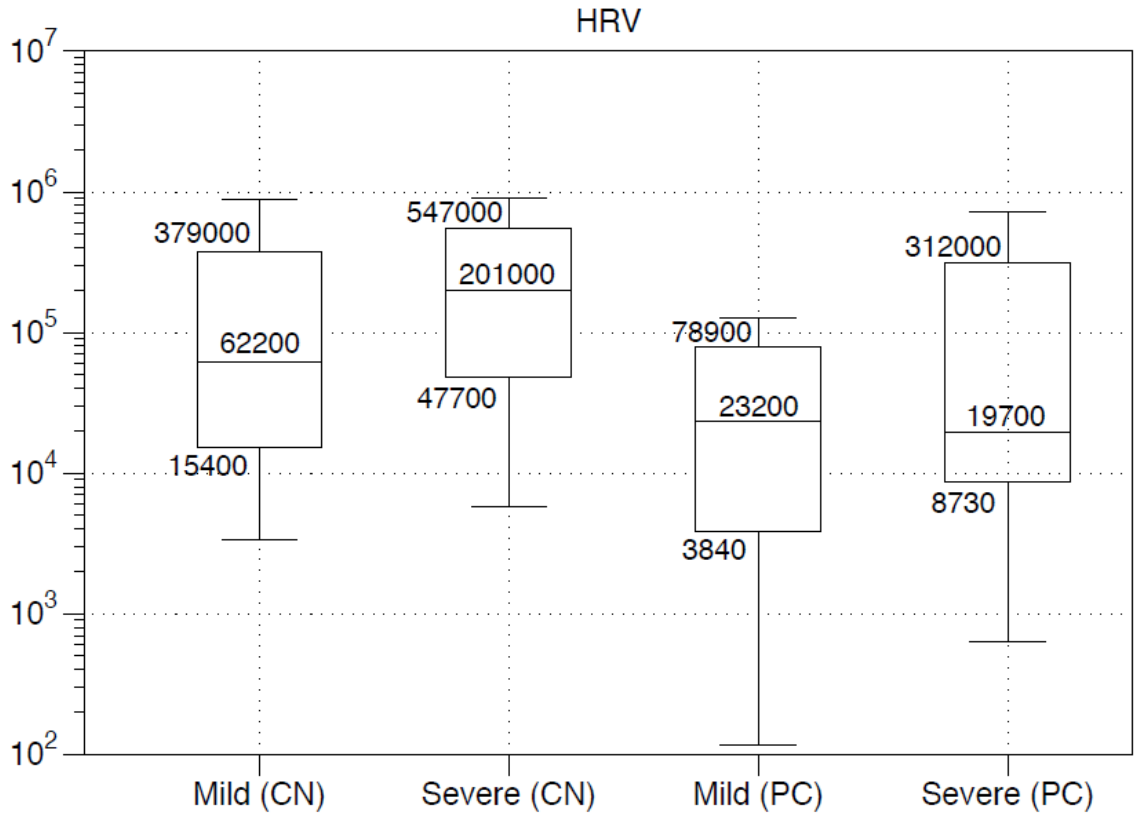


Figure 4.11. Quantification of human rhinovirus (HRV) RNA genome copies in nasopharyngeal aspirates using real-time RT-PCR according to the severity of disease. For mild pneumonia, median HRV copy number per µL [Mild (CN)] is 6.22 X 10^4 with a value of 3.79 X 10^5 at the 75th quartile mark and a value of 1.54 X 10^4 at the 25th quartile mark. For severe pneumonia, median HRV copy number per µL[Severe (CN)] is 2.01 X 10^5 with a value of 5.47 X 10^5 at the 75th quartile mark and a value of 4.77 X 10^4 at the 25th quartile mark. For mild pneumonia, median HRV normalised copy number per cell [Mild (PC)] is 2.32 X 10^4 with a value of 7.89 X 10^4 at the 75th quartile mark and a value of 3.84 X 10^3 at the 25th quartile mark. For severe pneumonia, median HRV normalised copy number per cell [Severe (PC)] is 1.97 X 10^4 with a value of 3.12 X 10^5 at the 75th quartile mark and a value of 8.73 X 10^3 at the 25th quartile mark. CN denotes copy number and PC denotes copy number per cell.

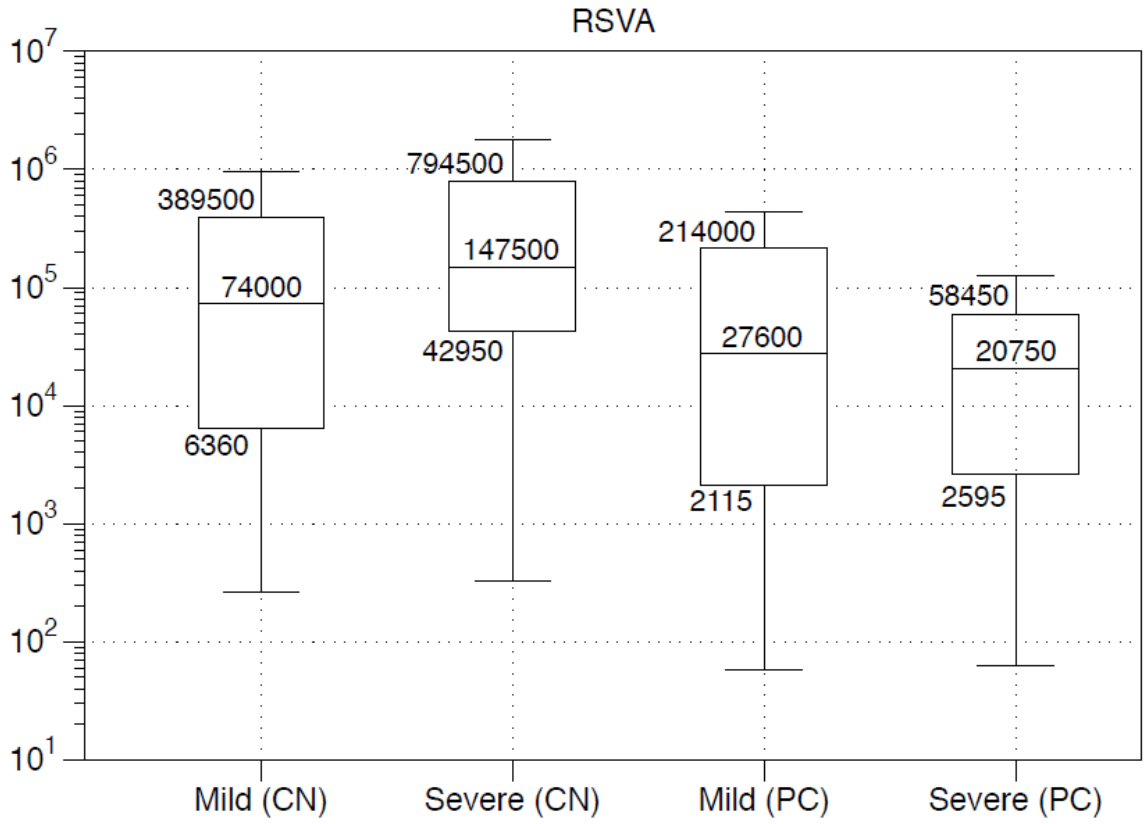


Figure 4.12. Quantification of respiratory syncytial virus A (RSV A) RNA genome copies in nasopharyngeal aspirates using real-time RT-PCR according to the severity of disease. For mild pneumonia, median RSV A copy number per µL [Mild (CN)] is 7.40 X 10^4 with a value of 3.90 X 10^5 at the 75th quartile mark and a value of 6.36 X 10^3 at the 25th quartile mark. For severe pneumonia, median RSV A copy number per µL[Severe (CN)] is 1.48 X 10^5 with a value of 7.95 X 10^5 at the 75th quartile mark and a value of 4.30 X 10^4 at the 25th quartile mark. For mild pneumonia, median RSV A normalised copy number per cell [Mild (PC)] is 2.76 X 10^4 with a value of 2.14 X 10^5 at the 75th quartile mark and a value of 2.12 X 10^3 at the 25th quartile mark. For severe pneumonia, median RSV A normalised copy number per cell [Severe (PC)] is 2.08 X 10^4 with a value of 5.85 X 10^4 at the 75th quartile mark and a value of 2.60 X 10^3 at the 25th quartile mark. CN denotes copy number and PC denotes copy number per cell.

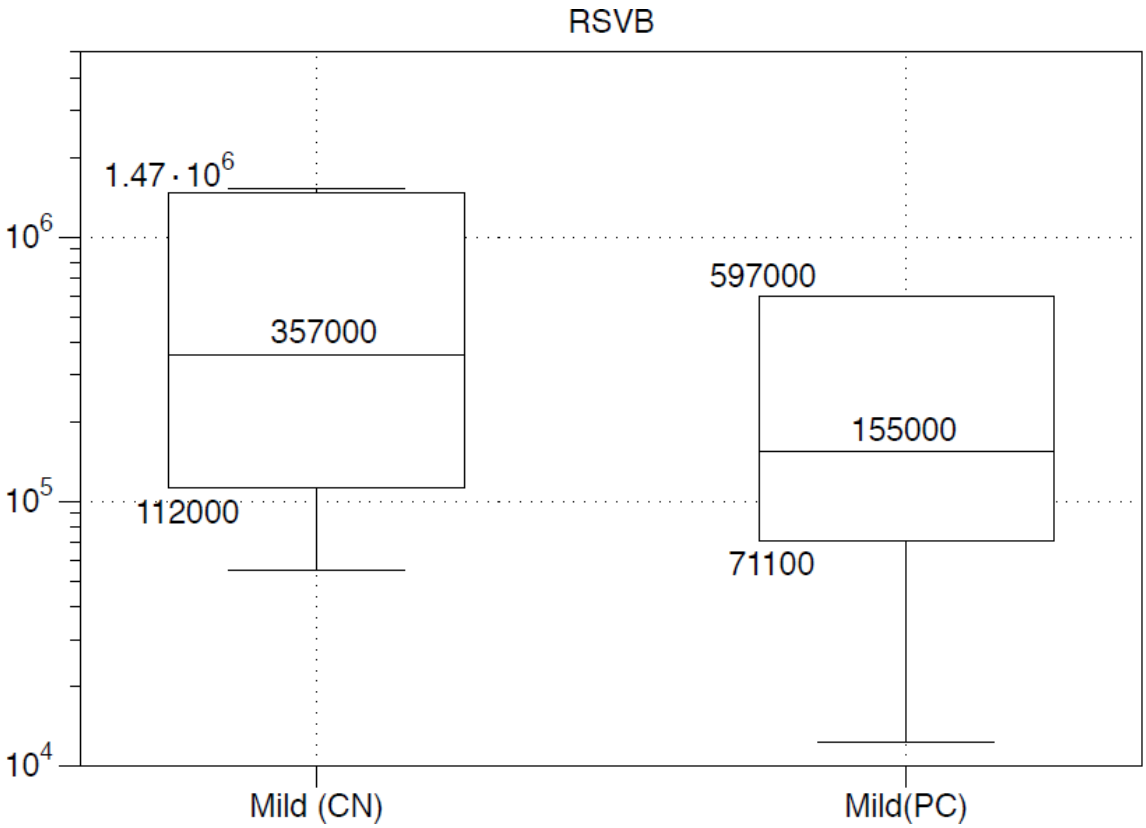


Figure 4.13. Quantification of respiratory syncytial virus B (RSV B) RNA genome copies in nasopharyngeal aspirates using real-time RT-PCR according to the severity of disease. The median RSV B copy number per µL[Mild (CN)] is 3.57 X 10^5 with a value of 1.47 X 10^6 at the 75th quartile mark and a value of 1.12 X 10^5 at the 25th quartile mark. For normalised copy number per cell [Mild (PC)], the median RSV B is 1.55 X 10^5 with a value of 7.11 X 10^4 at the 75th quartile mark and a value of 5.97 X 10^5 at the 25th quartile mark. CN denotes copy number and PC denotes copy number per cell

For hMPV, viral copy per µL ranged from 6.44 X 10^2 to 2.08 X 10^7 with a median value of 1.96 X 10^4 and normalized viral copies per cell ranged from 1.68 X 10^1 to 1.52 X 10^6 with a median value of 3.02 X 10^3 for mild pneumonia. As for severe pneumonia, viral copy per µL ranged from 9.28 X 10^2 to 1.39 X 10^5 with a median value of 1.93 X 10^3 and normalized viral copies per cell ranged from 1.31 X 10^3 to 1.12 X 10^5 with a median value of 6.76 X 10^3 (Figure 4.14). Based on median viral copies/ µL, only slight increases in viral load were observed for HRV, RSV A and RSV B in severe pneumonia. The only exception was hMPV where a slight decrease from 1.96 X 10^4 to 1.93 X 10^3 was recorded. However, after normalization with number of cells collected, there were no longer differences in the median viral copy numbers between mild and severe pneumonia (Table 4.5).

4.3.5 Viral Seasonality And Severity Of Disease

One of the earliest studies that suggested a seasonal pattern to respiratory viral infections of humans in the tropics was carried out on individuals living on an isolated island in the West Indies. In that study, the authors examined the incidence of "colds", which were essentially acute respiratory tract infections, in infected individuals and found an association of respiratory infections with a decrease in environment temperature, with an almost complete absence of colds during the hottest months of the year (Shek *et al.*,

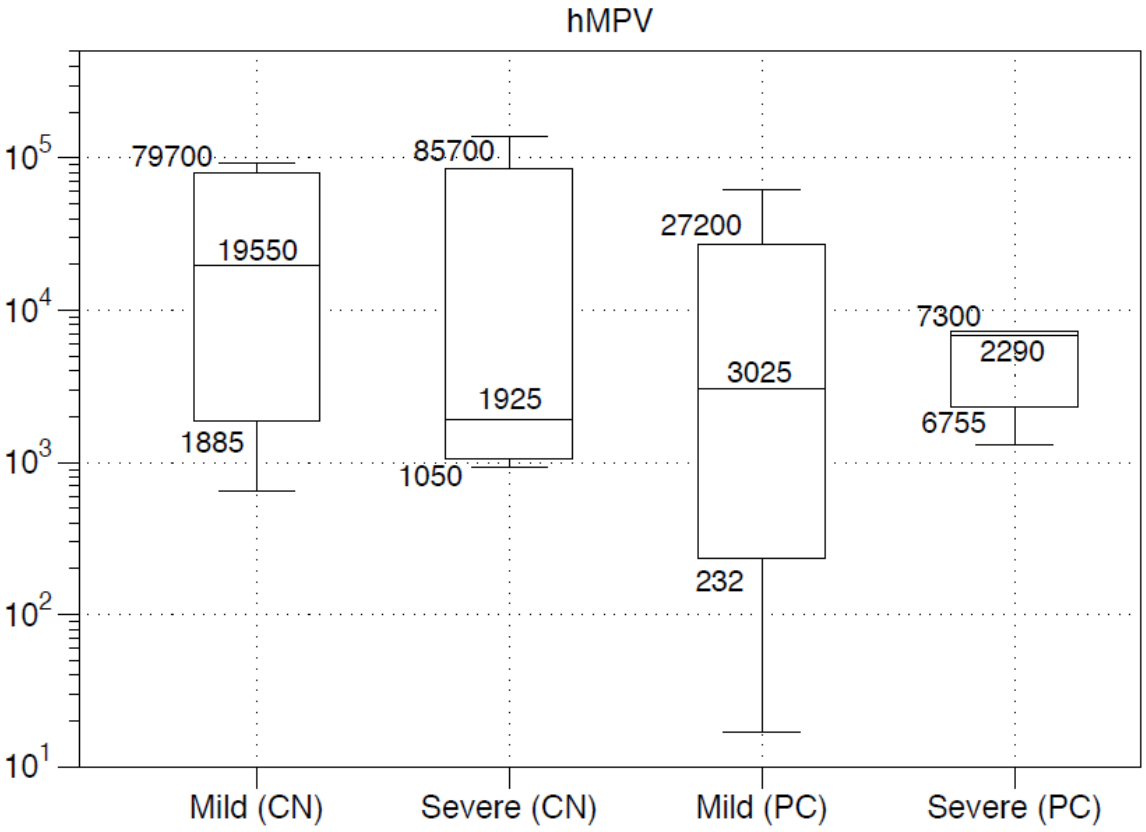


Figure 4.14. Quantification of human metapneumovirus (hMPV) RNA genome copies in nasopharyngeal aspirates using real-time RT-PCR according to the severity of disease. For mild pneumonia, median hMPV copy number per µL [Mild (CN)] is 1.96 X 10^4 with a value of 7.97 X 10^4 at the 75^{th} quartile mark and a value of 1.89 X 10^3 at the 25^{th} quartile mark. For severe pneumonia, median hMPV copy number per µL[Severe (CN)] is 1.93 X 10^3 with a value of 8.57 X 10^4 at the 75^{th} quartile mark and a value of 1.05 X 10^3 at the 25^{th} quartile mark. For mild pneumonia, median hMPV normalised copy number per cell [Mild (PC)] is 3.03 X 10^3 with a value of 2.72 X 10^4 at the 75^{th} quartile mark and a value of 2.32 X 10^2 at the 25^{th} quartile mark. For severe pneumonia, median hMPV normalised copy number per cell [Severe (PC)] is 6.76 X 10^3 with a value of 7.30 X 10^3 at the 75^{th} quartile mark and a value of 2.29 X 10^3 at the 25^{th} quartile mark. CN denotes copy number and PC denotes copy number per cell.

2003). Unlike the temperate regions of the world, temperature in the tropics is higher with less variability in seasonal changes. However, these regions do experience local variations in temperature, humidity and rainfall. Even though tropical countries are buffered from the harsh conditions of winter, the toll of respiratory infections is still high. Thus, one aspect of the current study was to gain a better understanding of the duration of RSV, hMPV and HRV infections in Bandung, Indonesia and to determine which meteorological conditions if any might affect the activity of these respiratory viruses in local communities. In addition, an attempt was made to correlate seasonality with disease severity.

A total of 352 episodes of pneumonia were diagnosed between the period February 1999 to May 2001. For the year 1999, cases of pneumonia were relatively low with occurrence between zero to six episodes per month with two small peaks observed between February to June and August to December. However, in 2000, a small outbreak was observed with the number of pneumonia peaked at 13 cases in May before decreasing to four cases in early September of the same year. A second peak was also observed when cases increased in late September before decreasing in December. For the year 2001, the number of pneumonia cases was relatively stable and occurred between one to seven cases per month. As specimens were only collected until May 2001, it was difficult to comment if the distribution of cases were similar to that of the year 1999.

Generally, HRV was detected almost throughout the 28 month period of sample collection (Figure 4.15). Cases detected were also consistent and fell between one to four episodes of infection per month. As for RSV, consistent annual peaks between late January to June were observed over a 28 month period, especially in the year 2000 and 2001. As noted in other studies (Zlateva *et al.*, 2007; Frabasile *et al.*, 2003), RSV B circulated concurrently with RSV A in each epidemic period. However, RSV A strains were isolated more frequently than RSV B. For hMPV, fairly constant detections between one to four episodes were noted between the year 1999 until the second quarter of 2000 where after which no cases were detected for the next two quarters. However, a sudden increase in the detection of hMPV episodes was noted in the first quarter of 2001 with a total of eleven episodes and for second quarter of 2001, a total of eight episodes were identified. In an attempt to determine whether or not a surge of episodes was related to weather patterns, statistical analyses were conducted initially using Pearson's correlation. However, as no linearity was observed for the data using Pearsons correlation, a non-linear Spearman correlation was subsequently used. Spearman correlation between weather parameters and RSV, hMPV and HRV incidence rates and proportion of severe pneumonia cases with lags of up to 5 weeks were conducted. However, after accounting for auto-correlation, no significant correlation between the weather parameters and disease

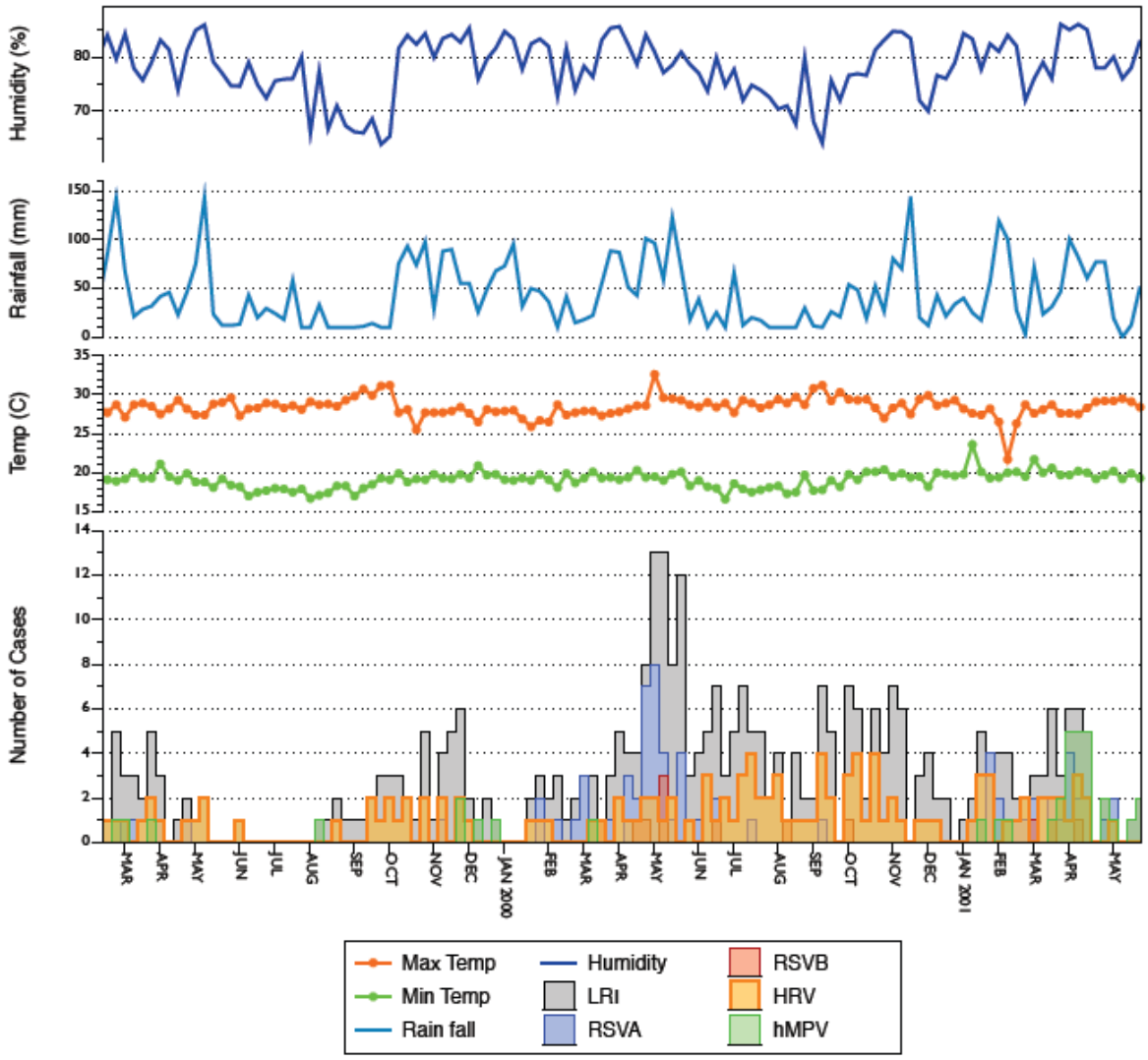


Figure 4.15. Seasonality of lower respiratory tract infection (LRI), RSV, hMPV and HRV positive samples collected over a 28 month period. HRV: human rhinoviruses, hMPV: human metapneumovirus, RSV A: respiratory syncytial virus A, RSV B: respiratory syncytial virus B and HRV, human rhinovirus.

incidence were detected. Furthermore, there were also no significant correlations detected between weather parameters and the proportion of severe cases at any lag (weather conditions were compared by using a lag of one week interval for up to 5 weeks and correlated to disease severity) (Table 4.6).

Table 4.6. Spearman correlation between weather parameters and RSV, HRV, hMPV incidence rates (with first-order differencing to account for possible autocorrelation) and proportion of severe cases with lags up to 5 weeks. *P*-values are shown in parentheses.

hMPV	t	t+1	t+2	t+3	t+4	t+5
MaxTemp	0.004 (0.969)	-0.014 (0.889)	-0.019 (0.852)	0.108 (0.290)	-0.077 (0.450)	-0.152 (0.136)
MinTemp	-0.043 (0.674)	0.105 (0.302)	0.022 (0.826)	0.046 (0.650)	-0.025 (0.806)	0.059 (0.562)
Rainfall	-0.065 (0.524)	-0.129 (0.206)	-0.119 (0.245)	0.005 (0.958)	-0.039 (0.701)	0.110 (0.281)
Humidity	0.0378 (0.712)	-0.121 (0.237)	-0.102 (0.318)	-0.095 (0.352)	-0.020 (0.848)	0.0703 (0.491)

HRV	t	t+1	t+2	t+3	t+4	t+5
MaxTemp	-0.041 (0.686)	0.035 (0.734)	0.044 (0.667)	0.050 (0.625)	-0.079 (0.444)	0.124 (0.223)
MinTemp	0.085 (0.406)	-0.107 (0.295)	0.040 (0.701)	-0.072 (0.481)	-0.014 (0.894)	-0.094 (0.356)
Rainfall	-0.006 (0.956)	-0.032 (0.751)	-0.124 (0.224)	-0.001 (0.993)	-0.025 (0.810)	-0.106 (0.299)
Humidity	-0.081 (0.430)	0.030 (0.767)	-0.160 (0.116)	-0.016 (0.879)	-0.046 (0.655)	-0.072 (0.479)

RSV	t	t+1	t+2	t+3	t+4	t+5
MaxTemp	-0.031 (0.759)	-0.100 (0.328)	0.028 (0.786)	-0.108 (0.291)	0.046 (0.655)	-0.068 (0.503)
MinTemp	-0.078 (0.446)	0.121 (0.235)	-0.203 (0.045)	-0.010 (0.924)	0.0405 (0.692)	-0.146 (0.152)
Rainfall	-0.128 (0.209)	0.068 (0.508)	-0.114 (0.262)	0.0871 (0.394)	-0.107 (0.294)	-0.050 (0.622)
Humidity	-0.072 (0.481)	0.065 (0.528)	-0.082 (0.423)	0.010 (0.921)	-0.116 (0.257)	0.010 (0.924)

Severity	t	t+1	t+2	t+3	t+4	t+5
MaxTemp	-0.051 (0.619)	-0.132 (0.197)	-0.023 (0.825)	-0.070 (0.493)	0.038 (0.709)	0.029 (0.776)
MinTemp	-0.034 (0.741)	0.040 (0.693)	-0.047 (0.647)	0.089 (0.384)	0.007 (0.944)	0.006 (0.954)
Rainfall	-0.122 (0.233)	0.060 (0.559)	0.024 (0.818)	-0.027 (0.792)	0.070 (0.491)	-0.102 (0.316)
Humidity	-0.017 (0.867)	0.071 (0.489)	0.029 (0.778)	-0.057 (0.576)	0.083 (0.415)	-0.072 (0.483)

HRV: human rhinoviruses, hMPV: human metapneumovirus, RSV: respiratory syncytial virus and Severity: severe pneumonia.

4.4 Discussion

4.4.1 Viral Etiology

In the present study, 57.1% of pneumonia had at least one of three viral pathogens detected: human metapneumovirus (hMPV), respiratory syncytial virus (RSV A and RSV B) or human rhinovirus (HRV). This prevalence (57.1%) is slightly lower than the 62% and 71% reported by Juven *et al.* (2000) and Drummond *et al.* (2000). However, the 57.1% of the present study is not surprising as the afore-mentioned studies included other frequently isolated viruses in childhood CAP for example: influenza A and B, parainfluenza type 1, 2 and 3 as well as adenovirus (Drummond *et al.*, 2000; Juven *et al.*, 2000). The most common of these three respiratory viruses investigated in the Indonesian birth cohort was HRV at 33.8% whilst RSV was the second most common virus detected in 24.1% of all 352 episodes screened. This is in agreement with another study conducted by Arden *et al.* (2006) where HRVs were the most frequently identified virus in pediatric acute respiratory tract infection (ARTIs). However, many other pneumonia studies found RSV to be the major pathogen accounting for between 14 and 40% of viral isolations from infants and preschool children (McIntosh, 2002, Mahony, 2008). In developed nations, RSV is found to be the largest single pathogen resulting in hospitalisation (Sinaniotis, 2004) whilst in the

developing countries, it is a major cause of pneumonia in children (Robertson *et al.*, 2004; Sinaniotis, 2004). The viruses that frequently cause pneumonia in children are listed in Table 4.7.

Human rhinoviruses (HRV) are probably one of the most common human pathogenic microorganisms accounting for up to 80% of common cold illnesses (Turner, 2007). However, as the infection caused by these viruses is deemed mild (Turner, 2007; Mackay, 2008) and since testing is not always available, routine screening for HRV strains occurs relatively infrequently. Thus, many studies conducted do not use assays that detect for the presence of HRVs as they do not identify these viruses as a common cause of ARTIs. With the development of better methodologies for viral detection, it is now becoming increasingly clear that the relatively benign nature of HRVs is only partly true as their roles in wheezing, asthma and lower respiratory tract infections including pneumonia in infected children has only recently been appreciated (Turner, 2007; Papadopoulos, 2004; Miller *et al.*, 2007).

Table 4.7. Viruses associated with pneumonia in children.

Viruses	**Developed * Countries (%)**	**Developing * Countries (%)**	**Our study (%)**
RSV	29 (24-63)	36 (26-78)	24.1
Parainfluenza			
type 1	9 (3-11)		
type 2	2		
type 3	9 (3-26)		
Total type 1-3	20	5 (3-13)	
Influenza			
type A	4 (3-9)	5 (4-13)	
type B	4	2 (1-6)	
Total type A-B	8	8	
Adenovirus	7 (6-7)	9 (3-48)	
HSV	3	4	
Rhinovirus	5 (2-25)		33.8
Enterovirus	5		
Human Metapneumovirus			8.5

* Adapted from Sinaniotis, 2004

Before the advent of PCR, a small number of studies looked for HRV by culture of specimens in childhood pneumonia (Juven *et al.,* 2000; Michelow *et al.,* 2004). These studies identified HRV in 2 to 10% of cases (Juven *et al.,* 2000; Michelow *et al.,* 2004). It has been noted that the general detection rate of viruses in studies on the etiology of childhood pneumonia that have not included HRV in their analyses is 20 to 45%, whilst studies that have looked for HRV have reported a 31-60% detection rate (Papadopoulos, 2004; Turner, 2007) It is probable that the differences in detection rate percentages can be attributed to HRVs (Papadopoulos, 2004). More recently, a study has used a wide range of methods, including PCR to determine the etiology of CAP in children and successfully identified a potential causative agent in 85% of the patients where viruses accounted for 62% of the infections and HRV was one of the most common agents, at an incidence of 24 (Papadoulos, 2004).

In the present study, a total of 352 episodes of infections were documented for 256 pediatric patients with pneumonia. It is interesting to note that slightly more than a quarter of the patients had at least one episode of a recurrent pneumonia infection during the 28 month study period. Underlying illness is believed to be the main reason why recurrent infections occur in these children. The most frequent underlying causes include recurrent aspiration, followed by immunodeficiency, asthma and structural anomalies (Lodha *et al.,* 2002; Huerta *et al.,* 2005; Ciftci *et al.,* 2003). However, upon reviewing results obtained from children with more than four episodes of infections in

the 28 months, it was noted that all six patients had at least one episode of infection caused by HRV, though they tended to have mild pneumonia, especially when HRV was the only causative agent. Notably, three out of the four patients with subsequent hMPV or RSV A viral infections tended to have severe pneumonia. Could this indicate that HRV infections predispose children to subsequent severe pneumonia, especially if the viruses are hMPV or RSV A? If this is so, it is not surprising as many studies have associated HRV with childhood wheezing and asthma leading to hospitalization (Korppi *et al.*, 2004; Lemanske *et al.*, 2005; Malmstrom *et al.*, 2006; Miller *et al.*, 2007). This, in turn predisposes patients to other respiratory virus infections

Whether the identification of HRV indicates causality, particularly for LRTI, remains controversial. Results of the present study are supportive of the notion that HRVs are implicated in childhood pneumonia and possibly may predispose patients to severe infections. However, an interesting observation reported in several studies is the possibilities that HRV detection may have been incidental in many of the cases diagnosed by upper respiratory specimens, because of either asymptomatic colonization or precedent infection. These studies have identified high prevalences, from 5% to 18% of HRV in the nasopharynx of asymptomatic children (Hayden *et al.*, 2004; Louie *et al.*, 2009). Also, the more important question of whether or not these infections could and/or should be a target of therapy should also be explored. With current therapeutic strategies relatively unavailable for many

populations, the question to be asked is should attempts be intensified to investigate potential treatment candidates? Before we can answer this question, it is probably most important to conduct more epidemiological studies to evaluate the prevalence of HRV in different populations and age groups and also to have a better understanding of the disease burden resulting from this infection. Importantly, the frequencies of these viruses in healthy babies should be incorporated as a control group.

Recent advances in molecular biology, particularly the introduction of the PCR assay, have greatly improved the detection of viral respiratory pathogens (Mahony, 2008). Yet, even using the most sensitive molecular techniques, only 40-60% of respiratory infections in paediatric populations have an associated causative microorganism (Louie *et al.*, 2005). This suggests that additional respiratory pathogens are likely to exist in these infection scenarios (van den Hoogen *et al.,* 2001), although it may also mean that sampling techniques are inadequate. Importantly, with the sensitivity of PCR technologies, one should note that the detection of viruses in respiratory specimens does not automatically infer infection, rather it indicate the presence of pathogens which are potentially associated with the illness.

Since 2001, seven previously undescribed viruses have been identified by analysis of clinical specimens from the human respiratory tract. These are human metapneumovirus (hMPV) in 2001 (van den Hoogen *et al.,* 2001),

three new human coronaviruses (HCoV), the severe acute respiratory syndrome (SARS) associated coronavirus in 2003 (Ksiazek *et al.*, 2003), coronavirus NL63 (NL63) in 2004 (van der Hoek *et al.*, 2004), coronavirus HKU1 (HKU1) in 2005 (Woo *et al.*, 2005a), as well as human bocavirus (HBoV) in 2005 (Allander *et al.*, 2005) and the recently described human polyomaviruses KI (KIV) and WU (WUV) in 2007 (Allander *et al.*, 2007a; Gaynor *et al.*, 2007). These new viral agents were detected by novel molecular methods such as VIDISCA (van der Hoek *et al.*, 2004), pan-viral DNA microarrays (Wang *et al.*, 2003), and high-throughput sequencing (Allander *et al.*, 2005, 2007a; Gaynor *et al.*, 2007).

Human metapneumovirus (hMPV) is a relatively new pathogen of the human respiratory tract first described by van den Hoogen in 2001. The virus was first isolated by cell culture from respiratory secretions collected during a 20-year period from Dutch children with ARTIs (van den Hoogen *et al.*, 2001). It was established that hMPV was a respiratory pathogen originating in primates, and resulting in mild upper respiratory tract symptoms of infected species. In humans, many studies have shown that the signs and symptoms of hMPV infection in infants resemble those induced by RSV infection (van den Hoogen, 2004; Kahn, 2006) and this causative virus is second only to RSV as a cause of bronchiolitis in early childhood (Boivin *et al.*, 2003; Chano *et al.*, 2005; Kahn, 2006). Children younger than 5 years of age are most susceptible to hMPV infection, and those younger than 2 years of age are most likely to

be hospitalized with severe symptoms, including bronchiolitis, pneumonia, and bronchitis (Kahn, 2006). More recently, it was shown that acute otitis media developed in 61% of hMPV-infected children younger than 3 years of age (Ljubin-Sternak, 2008; Sloots *et al.,* 2008). It is believed that hMPV accounted for up to 10% of respiratory infections in children under the age of 5 years, and has since been identified in Australia, the United Kingdom, North America, Asia (including Japan, Korea and China) and Southeast Asia (including Singapore and Thailand) (Kahn, 2006; Gillim-Ross *et al.*, 2006, Ong *et al.,* 2007, Loo *et al.,* 2007 and Thanasugarn *et al.*, 2003). The present study is, however, the first study to detect hMPV in an Indonesia pediatric population. Results obtained in this study have confirmed that 8.8% of all pneumonia cases investigated had hMPV. This prevalence is comparable with those of other studies (Sloots *et al.,* 2006; Mahony, 2008). Analyses of archived specimens from banked human sera suggested that hMPV has been circulating in the human population for at least 50 years (van den Hoogen *et al.,* 2001; Kahn, 2006). Thus, it is not surprising to find hMPV in a population especially if specific molecular detection techniques are employed to look for this virus as shown in this study.

One limitation of the study is that we did not look for other causative organisms of pediatric pneumonia. Respiratory viruses, other than RSV which are commonly associated with pneumonia include influenza, parainfluenza and adenovirus (Singh, 2005; Mathisen *et al*., 2010); whereas bacteria

pathogens may include *Streptococcus pneumonia*, *Haemophilus influenza* and *Mycoplasma pneumonia* (Singh, 2005). In endemic areas, malaria could also be another confounding infection mimicking a lower respiratory infection, although this risk is considerably lower in Indonesia (Robertson *et al*., 2004). Thus, further studies are warranted to determine the prevalence of these pathogens in respiratory samples and investigate their role as a causative agent in paediatric pneumonia.

4.4.2 Viral Seasonality

Indonesia is a large country situated around the equator and which experiences two monsoon seasons annually (Omer *et al.,* 2008). From November to March, the country is dominated by the north monsoon, blowing from China; while May to September is the period of the south monsoon, which blows from the Indian Ocean and Australia. A transition period between the two monsoons may be observed in April and October, during which the winds are light and variable in direction (Omer *et al.,* 2008; http://app2.nea.gov.sg/asiacities_indonesia.aspx). In our study of samples from patients that were situated in Bandung, Indonesia, RSV season occurred in late January and peaked between March to May before declining in June. This typically coincided with the north monsoon season which brings heavy rainfall and higher humidity. Association between RSV infection rates

and the rainy season were also observed in other studies conducted in Asia including Indonesia, Africa and South America (Shek *et al.*, 2003; Omer *et al.*, 2008). Possible reasons for this phenomenon include a reduction of UVB ray together with the presence of high humidity which prolonged survival of RSV during these rainy seasons. It is also speculated that children might be kept indoors more often and the resultant crowding may account for the increased prevalence as well (Shek *et al.*, 2003; Welliver 2007). Cases of RSV began to dip in June and this coincided with the beginning of the south monsoon which brought drier weather and lower humidity.

For human metapneumovirus (hMPV), circulation in temperate climates predominated in the late winter and spring, and the peak of activity at any given location often coincides with or follows the peak of RSV isolation (Kahn,2006; Williams *et al.*, 2006; Ljubin-Sternak *et al.*, 2008). In many communities, hMPV has been detected throughout the year, albeit at lower levels during the late spring, summer, and fall (Kahn, 2006; Ljubin-Sternak *et al.*, 2008). For the present study, small numbers of cases (one to four) were detected almost throughout the collection period for hMPV between February 1999 to April 2000 and after which, no cases were detected for a 6 month period. However, a sudden increase between January to May with peaks observed in March and April were noted for the year 2001. This might signify a small community outbreak during the study period or as noted by other studies, hMPV though causes annual community-wide epidemics of

respiratory tract disease, the rates of infections however differ somewhat from year to year (Alto *et al.*, 2004; van den Hoogen *et al.*, 2004). For the seasonal peaks of hMPV infections, many groups had reported similar RSV peaks between the late winter and early spring months, however it was noted that the greatest number of hMPV infections occurred approximately two months after the RSV peaks (Alto *et al.*, 2004; Ordas *et al.*, 2006; Williams *et al.*, 2006). In our study, we observed that the circulations of hMPV coincided with circulations of RSV and that they both peaked in the same March to May period for the year of 2001. With fewer seasonal changes and less variations in temperature and humidity than the temperate regions, it is possible that reasons that accounted for an increase in RSV circulations might be the same reasons why hMPV prevails in those months, especially when the disease patterns of RSV and hMPV are supposedly similar.

It was reported that rhinovirus infections are less affected by seasonality and tend to be evenly distributed throughout the year which was also observed in our study (Mackay *et al.*, 2008; Cooper *et al.*, 2007). However, some studies noted rhinovirus to be seasonal which tend to peak in the spring and autumn months (Miller *et al.*, 2007; Monto *et al.*, 2002). The prevalence of this infection could be due to the ease of viral transmission and the hardiness of this virus. It was noted that HRVs can retain infectivity for hours to days on suitable surfaces thus supporting the practical possibility of self-inoculation via fomites (Mackay *et al.*, 2008).

4.4.3 Factors Associated With Disease Severity

Community-acquired pneumonia (CAP) is an important cause of considerable morbidity and mortality, and requires hospitalization of patients worldwide (Feldman, 2004). CAP places an enormous clinical and financial burden on the healthcare system. In the developed world, an estimated worldwide incidence of 10 million cases annually, with a mortality rate as high as 50% in seriously ill patients and in the high-risk cases were documented (Feldman, 2004). In the United States, there are an estimated 5 million cases of CAP annually, accounting for more than 10 million visits of patients to the physician, 10% of which require hospitalization (Brixner, 2004). CAP is the sixth leading cause of death (3.5% of all deaths) in the United States and is the most frequent infectious cause of death among patients of all ages (46% of all infectious deaths) (Brixner, 2004). For the developing countries, the estimated number of paediatric deaths from CAP is 3 million with countries in Africa and Asia recording 2-10 times more children with pneumonia than the United States (Singh, 2005).

The sheer magnitude of CAP, with its association with morbidity and mortality rates, places a heavy economic burden on the healthcare system. The main therapeutic management of CAP currently is through the use of empirical antibiotic treatments (Lichenstein, 2003; Feldman, 2004). In most of these cases, antibiotic use is neither warranted nor associated with any

significant benefit as viruses account for up to 90% of pneumonia during the first year of life, though this percentage decreases to approximately 50% by school age (Kafetzis, 2004). One aspect to better management of CAP treatment therapy is not to simply provide a convenient diagnostic label for illness and providing a prescription, but attempting to make as accurate a diagnosis as possible on clinical and if necessary laboratory parameters, and then truly assessing the need for antibiotics and the likelihood that they would be of benefit in any particular case, balancing benefits with possible side effects. An early intervention through the use of vaccines has also been shown to be beneficial in reducing the burden of pneumonia (Burgner *et al.*, 2005; Farha *et al.*, 2005; Fuchs *et al.*, 2005).

Current immunisation strategies have decreased the number and severity of CAP infections and newer vaccines may bring considerable further benefits. However, prior to vaccine developments, issues to address include determining etiological agents in CAP, understanding interrelationships between risk factors, mixed infections, subgroup, viral load, and disease severity which are all important for the intervention strategies against these pathogens. Thus, this present study was performed to characterize these relationships for common etiological agents of childhood viral pneumonia: RSV, hMPV and HRV where no effective vaccines have been developed (Kahn, 2006; Mackay, 2008).

One limitation of our study is that we did not take into account known host and environmental risk factors for LRTI in regression analyses. Examples of host factors include gestational age, low birth weight, under-nutrition and lack of breastfeeding while environmental factors include air pollution (tobacco smoke) and overcrowding (Singh, 2005). These factors together with viral factors could potentially be the cause of disease severity and should warrant carefully analyses for their contributions to children contracting severe pneumonia. Only when the risk factors for severe disease can be identified can strategies be in place to prevent and/or reduce the severity of the infections.

4.4.3.1 Mixed Infections

Mixed respiratory viral infections are controversial and their association with severe disease is supported by some groups (Semple *et al*., 2005; Calvo *et al*., 2008). These varied observations can be explained by the lack of sensitivity of the assessed methods used for viral identification and by the small number of patients included in the randomised cohort studied (Paranhos-Baccala *et al.*, 2008). With the rapid development of molecular amplification techniques, we are now able to achieve better viral detection together with the ability to determine multiple infections through the use of a single patient specimen, thus enhancing our knowledge of the viral epidemiology of respiratory tract

infections. For the current study, the use of a microarray-based approach demonstrated the advantage of screening samples for the presence of multiple pathogens, which was later confirmed with the specific use of the real-time RT-PCR technique.

Various study groups have assessed the impact of mixed infections and their contribution to disease severity (van Woensel *et al.*, 2006; Wilkesmann *et al.*, 2006). Among the co-infections, the comparison of dual hMPV/RSV lower respiratory tract infection has been the most studied, and was associated either with no greater disease severity as observed in the current study or with an increased risk requiring the use of mechanical ventilation as reported by Semple *et al.*, (2005) as well as Richard *et al.*, (2008). More recently, it was described that RSV and HRVs are the viruses most frequently identified in dual infections in infants hospitalised with bronchiolitis. Their occurrence in bronchiolitis pathogenesis has been increasingly recognised with the establishment of new and highly-sensitive molecular amplification methods, especially for viruses that are difficult to culture (Paranhos-Baccala *et al.*, 2008).

For the current study, statistical significance (P value = 0.06) was achieved for co-infections of HRV and RSV A and their association in eight cases severe pneumonia. These children demonstrated lower chest indrawing with or without tachypnoea, severe lethargy, difficult to arouse or general danger

signs as in concordance with WHO classifications. However, in another study by Marguet *et al*. (2009), the role of HRV was established in infants with acute bronchiolitis and no additional effect of dual RSV/HRV infection on disease severity was observed. One reason for these discrepancies could be due to genetic predisposition. There are growing interests in the areas of candidate gene approaches where genes associated with immune response and their association with severe infections are being investigated (Miyairi *et al.*, 2008). A review by Miyairi and DeVincenzo (2008) stated that candidate gene approaches have demonstrated associations of severe infections in healthy infants with genetic polymorphisms that may alter the innate ability of humans to control RSV (surfactants, Toll-like receptor 4, cell surface adhension molecules, and others) and those that may control differences in proinflammatory responses or enhanced immunopathy (specific cytokines and their receptors). Thus, together with viral factors, genetic diversity of the host may reveal more complex relationships leading to disease severity. This may also explain why our cohort studied is more prone to severe pneumonia when infected with both RSV A and hMPV. However, to truly determine if genetic factors play a role in causing severe lower respiratory infection in this Indonesian cohort, we will need to perform a large scale genetic association study to demonstrate differences in immune responses due to single nucleotide polymorphisms (SNPs) in immune-related genes predisposing this population to disease severity. Currently, the few literature published on these genetic studies are mainly conducted in the United States and The

Netherlands (Kelly *et al.*, 2008; Miyairi *et al.*, 2008; Siezen *et al.*, 2009), who have populations that are genetically distinctive from the Indonesia population. In addition, to further clarify this point, the number of patients with co-infections of HRV and RSV A should also be enlarged and include both larger cohorts of mild as well as severe pneumonia cases for comparative study. Hopefully then, further information on the relevance of viral coinfection in respiratory tract infections can be revealed.

4.4.3.2 Viral load

Clinical severity of respiratory tract pathogens is likely to be the result of a combination of both host and viral factors (Fodha *et al.*, 2007; Rossi *et al.*, 2007). During infections, host factors such as young age (6 months), prematurity, low-birth weight, and chronic diseases have been associated with disease severity (Rossi *et al*., 2007; Olszewska *et al*., 2009). On the other hand, viral factors, such as viral load associated with disease severity are not understood sufficiently (Fodha *et al.*, 2007; Corsello *et al.*, 2008).

Following the use of quantitative RT-PCR in recent years, real-time RT-PCR has become the method of choice for quantification of viral load in nasopharyngeal aspirates (NPAs) taken during acute respiratory tract infections (Perkins *et al.*, 2005). Thus, using the Taqman® real-time RT-PCR

approach, viral load for RSV A, RSV B, hMPV and HRV were investigated and compared to disease severity in the present study. Comparisons between viral loads in terms of disease severity have produced controversial results: some studies reported an association between nasopharyngeal viral load and disease severity (Fodha *et al.*, 2007; Gerna *et al.*, 2008; Martin *et al.*, 2008) where as other authors failed to support such a conclusion (Wright *et al.*, 2002; van Elden *et al.*, 2008). Factors that could possibly explain these discrepancies include the choice of sample for the study of viral loads. With current practice, determinations of viral loads are usually done with samples from the upper respiratory tract, because lower respiratory tract secretions cannot be obtained reliably from non-intubated patients. Though this might not seem like a true reflection of the state of infection, previous studies have shown that the viral load of nasal washes closely approximates that found in tracheal aspirates (Malley *et al.*, 1998, 2000).

Another important factor to consider when determining viral loads is the time of sample collection as viral shedding may differ between the start of the infection and the point of recovery and nasopharyngeal aspirates may not always be taken at the peak of acute respiratory tract infections. It is known for RSV that viral shedding is the highest between 0 and 6 days and thus sampling should preferably be done in this period (Meerhoff *et al.*, 2010). However, as the design for this study is such that patients will be visited by kaders weekly and followed up every 2 days if they have pneumonia, the

chances of missing the highest viral shedding is thus very low. Instead, it is the variability in sample volume or quality that could most likely affect the measurement of viral load. Thus, to account for these variations in sample collection and to ensure the reproducibility of the assay, our current study uses 18S rRNA (a human housekeeping gene) as a control to normalise and standardise the non-homogenous respiratory samples and was tested in parallel with each assay. As compared to other commonly used housekeeping genes: β-actin mRNA, β-2 microglobulin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 18S rRNA was found to be the most stable housekeeping gene and hence more suitable for use as internal control in quantitative studies (Schmittgen *et al.*, 2000; Bas *et al.*, 2004). Prior to normalization, only slight increases in viral load were observed for HRV, RSV A and RSV B in severe pneumonia. However, after normalization with the number of cells collected using 18S rRNA, there were no statistical differences between the children with severe and mild pneumonia for all viruses tested including hMPV. Thus, severity of disease does not appear to be linked to viral load. This observation was also reported by Gueudin *et al.*, (2003) when normalisation of RSV load using GAPDH did not relate to disease severity.

CHAPTER 5 – MOLECULAR EPIDEMIOLOGY OF RSV A AND HMPV

5.1 Introduction

Antigenic variability is thought to contribute to the capacity of a virus to re-establish infections throughout life and is highly likely to pose an important challenge for vaccine design (Parveen *et al.*, 2006; Reiche *et al.*, 2009). Future planning for vaccine development will require an understanding of the genetic composition of the virus strains circulating among target populations. In this study, the genetic diversity of RSV A and hMPV strains collected in an Indonesian birth cohort study from young children with pneumonia was evaluated. Attempts were also made in the current study to correlate the genetic diversity of the two viral strains with severity of pneumonia. With more than 100 immunologically distinct serotypes identified, HRV genotyping was not included in this study due to their genetic complexities. RSV B subgroup was also not included in this genotyping exercise as there were too few strains collected from this cohort.

Respiratory syncytial virus (RSV) is the most commonly identified viral agent of acute lower respiratory tract infections (ALRTIs) of young children and causes repeat infections throughout life. Limited data are available on the molecular epidemiology of RSV from developing countries, including Indonesia (Parveen *et al.*, 2006). RSV strains vary genetically and antigenically and have been classified into two broad groups, A and B, with additional variability detected within the groups (Parveen *et al.*, 2006; Zlateva

et al., 2007; Reiche *et al.*, 2009). The RSV glycoprotein (G protein) is a type II integral membrane protein which showed the highest degree of divergence both between and within groups A and B (Zlateva *et al.*, 2007; Reiche *et al.*, 2009). The G protein is highly glycosylated, and it is the target of neutralizing and protective immune responses (Reiche *et al.*, 2009), and it has been suggested that antigenic differences within this protein could facilitate repeated RSV infections (Zlateva *et al.*, 2007; Reiche *et al.*, 2009). Variability in the G protein is concentrated in the extracellular domain, which consists of two hypervariable regions separated by a central conserved region of 13 amino acids. The second variable region, which corresponds to the C-terminal region of the G protein, reflects overall G protein gene variability and has been analyzed in molecular epidemiological studies (Parveen *et al.*, 2006; Zlateva *et al.*, 2007).

Epidemiological surveys have revealed that strains from both RSV groups can be present in the same community during outbreaks; however, their relative proportions maybe different between epidemics (Fodha *et al.*, 2007; Zlateva *et al.*, 2007; Reiche *et al.*, 2009). RSV has complex circulation patterns, with multiple genotypes or lineages co-circulating within the same community and replacement of the predominating genotypes with new ones over successive epidemic seasons (Sullender *et al.*, 2000; Viegas *et al.*, 2005; Zlateva *et al.*, 2007; Reiche *et al.*, 2009). RSV genotype distribution patterns can be distinctive for each community, and it has been suggested that they are

determined by local factors such as the level of herd immunity to certain strains (Zlateva *et al.*, 2007). Thus, in the present study, the genetic variability of the G protein from RSV A strains circulating in Bandung, Indonesia between the period February 1999 to May 2001 were determined and correlated with disease severity.

In the present study PCR assays were performed to type the gene encoding for the G proteins of all 73 strains of RSV A identified in the 352 nasopharyngeal samples collected from the paediatric patient cohort. However, after various attempts using different primer sets including sequencing of smaller gene fragments with another set of primers, only 21 strains were successfully amplified and subjected to sequencing. The unsuccessful amplification of the remaining 52 strains was later found to be due to degeneration of the RNA, which had been kept for up to ten years in the -80°C freezer. Fortunately, real-time RT-PCR assays for RSV A were not affected as they were done much earlier in 2004/2005 instead of in 2008 for sequencing reactions.

Human metapneumovirus (hMPV) is a newly discovered pathogen associated with respiratory infections ranging from upper respiratory infections to lower respiratory infections, such as bronchitis, bronchiolitis and pneumonia, and has been classified as a member of the genus *Metapneumovirus* of the subfamily *Pneumovirinae* of the family *Paramyxoviridae*, which also contains RSV (van den Hoogen., 2001; Bastien *et al.*, 2003; Alto *et al.*, 2004; Kahn,

2006; Camps *et al.*, 2008). RSV, the most closely related human pathogen to hMPV, and the genomes of the viruses have similar features as well as distinct differences. Both viruses are found to have the three putative viral envelop glycoproteins: the F (fusion), G (attachment), and SH (short hydrophobic) proteins, though the order of these genes differs between them (van den Hoogen *et al.*, 2002; Kahn, 2006). Of the three genes, the fusion (F) protein gene is the most highly conserved between the hMPV and RSV (van den Hoogen *et al.*, 2002; Kahn, 2006). Other than the gene order, one major difference is the lack of two non-structural genes, NS-1 and NS-2, at the 3' end of hMPV genome, while these 2 genes in RSV encode for anti-interferon activity, their functions in hMPV is unknown (Kahn, 2006). Using reverse genetics system, the M2 gene of hMPV carrying two overlapping open reading frames (ORFs), designated M2-1 and M2-2 are found to be expressed during viral replication. Recombination of hMPV lacking M2-2 gene was found to be attenuated in viral replication but retained adequate gene expression capabilities to induce protective immunity (Buchholz *et al.*, 2005).

Based on genetic and phylogenetic analyses, hMPV is separated into two subgroups A and B, with each subgroup divided into genotypes 1 and 2 (van den Hoogen *et al.*, 2004). Each season, several different hMPV genotypes co-circulate, with the predominant genotypes varying from season to season (Matsuzaki *et al.*, 2008). In 2004, a variant hMPV, isolated from a six and a half year-old girl with an acute exacerbation of asthma, was found to be

genetically distinct from viruses of the four lineages (A1, A2, B1 and B2) (Kahn, 2006). This strain was detected only with primers that targeted the N gene. In another study by Huck *et al.,* (2006), a novel hMPV lineage within A2 genotypes, A2a and A2b, were described. Thus, viruses in A genotype may be more divergent than previously appreciated.

RSV, which belongs to the same subfamily *Pneumovirinae* as hMPV, is also divided into groups A and B, and some studies have reported that subgroup A isolates are associated with more severe illness (Gilca *et al.,* 2006; Matsuzaki *et al.,* 2008). Although a few studies have reported differences in clinical severity between hMPV subgroups A and B, (Agapov *et al.,* 2006; Vicente *et al.,* 2006) the relationship between hMPV genotypes and severity remains controversial (Matsuzaki *et al.,* 2008). In a study by Vincente *et al.,* (2006), acute respiratory tract infections in small children due to hMPV genotype A were found to be more severe than infections due to genotype B. While other studies found group B to be associated more often with laryngitis and wheezing as compared to group A (Matsuzaki *et al.,* 2008), Agapov *et al.* (2006) found no differences between disease severities and hMPV genotypes. Thus, in our current study, the genetic variability of the F protein from hMPV strains circulating in Bandung, Indonesia between the period February 1999 to May 2001 were determined and correlated with disease severity.

5.2 Materials and Methods

5.2.1 RSV A Sequencing

Real time RT- PCR and sequencing primers for RSV A were designed with reference to the sequences in the NCBI GenBank (Appendix 6). cDNA was synthesized using the SuperScript™ III First-Strand Synthesis System (Invitrogen™, Carlsbad, CA, USA) following manufacturer's instructions with 0.5µM of RSVArtR primer and 5µL of viral RNA used for each reaction.

For amplification, *Pfu*Ultra® II Fusion HS DNA Polymerase (Stratagene©, La Jolla, CA, USA) was used with 2µL of cDNA in each reaction following the recommended protocol. The cycling conditions consisted of an activation step of 92°C for 2 minutes; 45 cycles of denaturation at 92°C for 10 seconds; annealing at 55°C for 20 seconds; elongation at 68°C for 1minute and a final 5 minutes extension at 68°C. Samples were then cooled to 4°C and stored at -20°C.

PCR products (897 bp) were separated on a 1% agarose Tris-boric-acid (TBE) gel and visualized under UV light with ethidium bromide staining. The band of interest was extracted and purified using the Qiagen QIAquick® PCR Purification Kit (Qiagen, Germany) with an elution volume of 30 µL. Purified

products were quantified using a Thermo Scientific Nanodrop™ ND-1000 Spectrophotometer.

For every sequencing reaction, approximately 40 ng of PCR product and 3.2 pmol of primer were added into a BigDye Terminator v3.1 master mix (Applied Biosystems, CA). The mixture was then subjected to 20 cycles of PCR and cleaned with CleanSEQ (Agencourt Bioscience, MA) before being analyzed using the capillary electrophoresis in the ABI 3730xl genetic analyzer (Applied Biosystems, CA). Sequences were aligned and analyzed using SeqScape v2.5 (Applied Biosystems, CA) and MEGA 3.1 program (Kumar *et al.*, 2004). Phylogenetic analysis was performed using the sequences obtained. In total, 21 samples were used to construct a neighbour-joining (NJ) tree for G gene using MEGA 3.1.

5.2.2 hMPV Sequencing

For sequencing, DNA templates were synthesized using the QIAGEN OneStep RT-PCR Kit (Qiagen, Germany). A 50 µL reaction mixture containing 2µL of a 1:10 diluted RNA, 3 µL of forward primer and 3 µL of reverse primer were used for the reaction. N-gene primers, hMPV_NP_F379 (forward): 5'-ATAGACAAAGARGCAAGAAAAAC -3' and hMPV_NP_R986 (reverse): 5'- GCATTRCCRAGAACAACACT -3', were

designed according to the hMPV sequences published in NCBI GenBank. As for the F-gene, hMPV-fg_F (forward): 5'- ATGTCTTGGAAAGTGGTG -3' and hMPV-fg_R (reverse): 5'- CCATGTAAATTACGGAGCT -3'; they were designed using Primer Express version 1.0 software (Applied Biosystens, USA). The thermal conditions consisted of a reverse transcription step at 50°C for 30 minutes; an initial PCR activation step at 95°C for 15 minutes; followed by a 3-step cycling condition at 94°C for 1 minute; 56°C for 1 minute; 72°C for 1 minute and a final extension step at 72°C for10 minutes for 40 cycles before cooling at 4°C. Forty-six partial N-genes and 35 partial F-genes were successfully amplified using this method. PCR products were separated on a 1% agarose TBE gel and visualized under UV light with ethidium bromide staining. The PCR products of about 608 bp for the N-gene and 811 bp for the F-gene were extracted and purified using Qiagen QIAquick® (Qiagen, Germany), with an elution volume of 30 µL. Purified products were quantified using a Thermo Scientific Nanodrop™ ND-1000 Spectrophotometer.

For every sequencing reaction, approximately 40 ng of PCR product and 3.2 pmol of primer were added into a BigDye Terminator v3.1 master mix (Applied Biosystems, CA). The mixture was then subjected to 20 cycles of PCR and cleaned with CleanSEQ (Agencourt Bioscience, MA) before being analyzed using the capillary electrophoresis in the ABI 3730xl genetic analyzer (Applied Biosystems, CA). Sequences were aligned and analyzed

using SeqScape v2.5 (Applied Biosystems, CA) and MEGA 3.1 program (Kumar *et al.*, 2004). Phylogenetic analysis was performed using the sequences obtained. In total, 36 samples were used to construct a neighbour-joining (NJ) tree for the N-gene and 26 samples were used to construct a separate NJ tree for the F-gene using MEGA 3.1

5.3 Results

5.3.1 Genetic diversity of RSV A

Twenty-one out of the 73 strains of RSV A isolated during the 28 months from a birth cohort in Indonesia were used for the study of genetic diversity. In order to detect the genetic variability of the 21 isolates, whole G protein genes (897 nt) were amplified and sequenced. However, to correlate results with previous reports of the RSV A strains from other studies, the sequences were further trimmed to only include the 3' terminal of the G protein gene at 264 nt between the position 634 to 897 for the analysis with 15 other published sequences from Genbank in NCBI.

Analysis of all 21 strains tested revealed that they belonged to three genotypes: 18 (85.7%) viruses to genotype GA5, two (9.5%) to genotypes GA2 and one (4.8%) to genotype GA7 (Figure 5.1). Phylogenetic analyses (MEGA 3.1) also revealed that GA5, the most predominant strain in this cohort, was also the prevailing subgroup A genotype during the epidemic season of the first and second quarters of 2000. As for GA 2, only two cases during the first quarters of 2000 and 2001 were detected for the 28 months of collection, while for GA7, only one case was detected in the first quarter of 2000 (Figure 5.2). Out of the 21 strains analysed, six episodes of infections

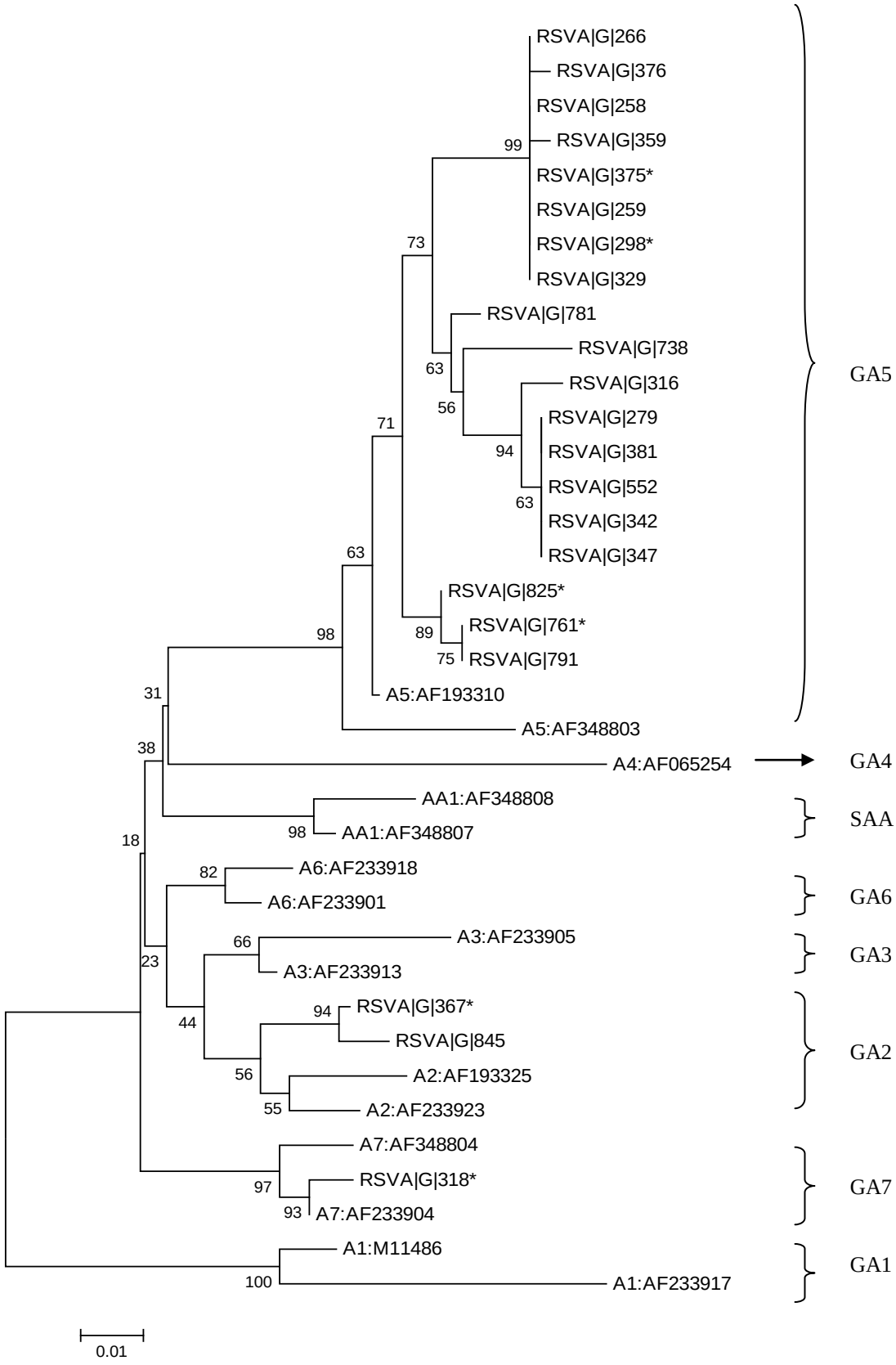

RSVA|G|266
RSVA|G|376
RSVA|G|258
RSVA|G|359
RSVA|G|375*
RSVA|G|259
RSVA|G|298*
RSVA|G|329
RSVA|G|781
RSVA|G|738
RSVA|G|316
RSVA|G|279
RSVA|G|381
RSVA|G|552
RSVA|G|342
RSVA|G|347
RSVA|G|825*
RSVA|G|761*
RSVA|G|791
A5:AF193310
A5:AF348803
A4:AF065254
AA1:AF348808
AA1:AF348807
A6:AF233918
A6:AF233901
A3:AF233905
A3:AF233913
RSVA|G|367*
RSVA|G|845
A2:AF193325
A2:AF233923
A7:AF348804
RSVA|G|318*
A7:AF233904
A1:M11486
A1:AF233917
99
73
63
56
94
63
71
63
89
75
98
31
38
98
18
82
66
23
44
94
56
55
97
93
100
GA5
GA4
SAA
GA6
GA3
GA2
GA7
GA1
0.01

Figure 5.1. Phylogenetic analysis of partial G gene of RSV A strains. Analysis was performed using 264 nt from the G gene for 21 RSV A strains collected from an Indonesian birth cohort between February 1999 to May 2001. A phylogenetic tree was constructed using the neighbour-joining method with 1,000 pseudoreplicate datasets. RSV A strains isolated are designated by an abbreviation RSVA|G| followed by their unique episode number and * indicate severe pneumonia. The nomenclature is based on phylogenetic clustering with sequences previously assigned to specific genotypes obtained from Parveen *et al.,* 2006 and designated by an abbreviation AF or M followed by their unique Genbank number.

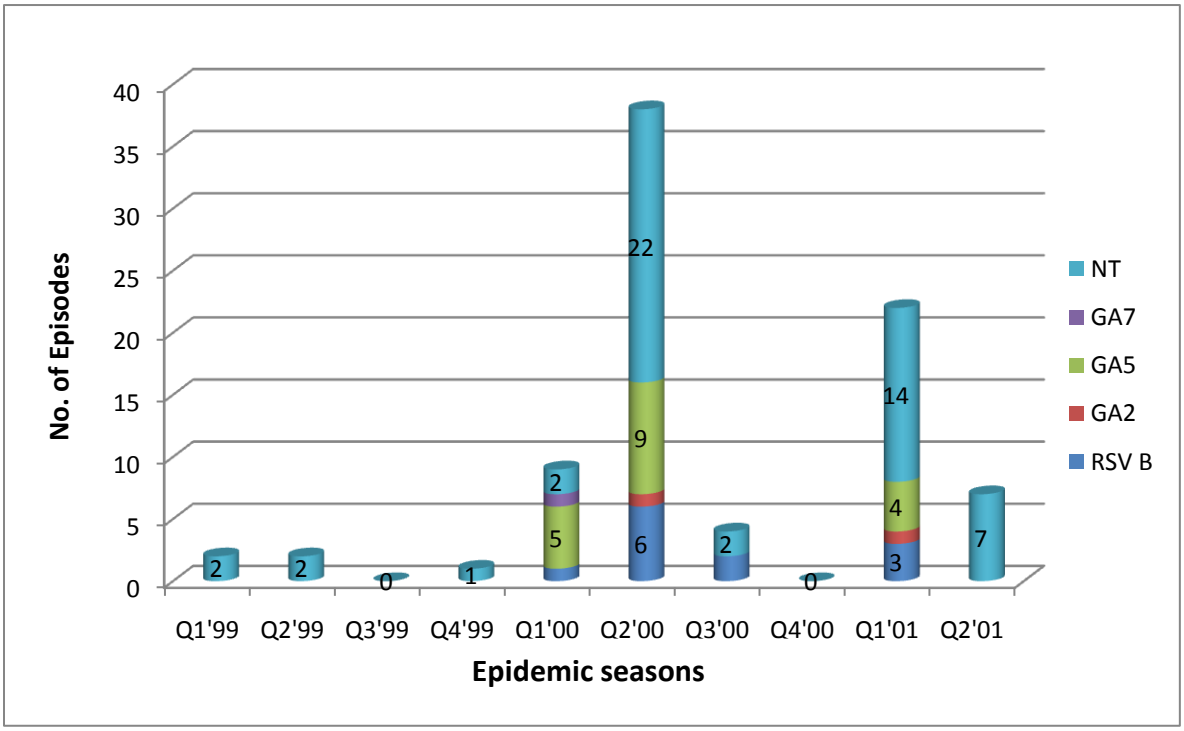


Figure 5.2. Seasonal frequencies of RSV A strains: GA2, GA5, GA7 and RSV B strains collected during a 28 month period. Twenty-one out of the 73 strains of RSV A isolated were used for the study of genetic diversity. GA5 was the most predominant strain and the most prevailing subgroup A genotype during the epidemic season of the first quarters of 2000 and 2001 and second quarter of 2000. NT denotes non-typeable RSV A strain. Q1' denotes January to March, Q2' denotes April to June, Q3' denotes July to September and Q4' denotes October to December. 99 denotes 1999, 00 denotes 2000 and 01 denotes 2001.

were associated with severe pneumonia: 298, 318, 375, 367, 761 and 825 (Figure 5.1). However, an attempt to correlate a specific and predominant strain in patients with severe pneumonia was unsuccessful as all three genotypes, i.e. GA2, GA5 and GA7 were identified in the severe group.

5.3.2 Genetic diversity of hMPV

All 30 strains of hMPV isolated from352 nasopharyngeal samples during the 28 months (February 1999 to May 2001) from the birth cohort in Indonesia were used for this portion of the present study. In order to detect the genetic variability of the isolates, partial F protein at 537 nt between positions 61 – 597 nt were amplified and sequenced. Of the 30 strains, successful F gene sequences were obtained for only 21 strains (Figure 5.3). Thus, to ensure all isolates are successfully sequenced for analysis, the N gene was next chosen as it is also one of the more conserved genes. All 30 isolates were finally successfully sequenced between positions 487 - 837 nt (350 nt). Sequences for the N gene were analysed together with 17 other published sequences from Genbank in NCBI to generate a phylogenetic tree (Figure 5.4).

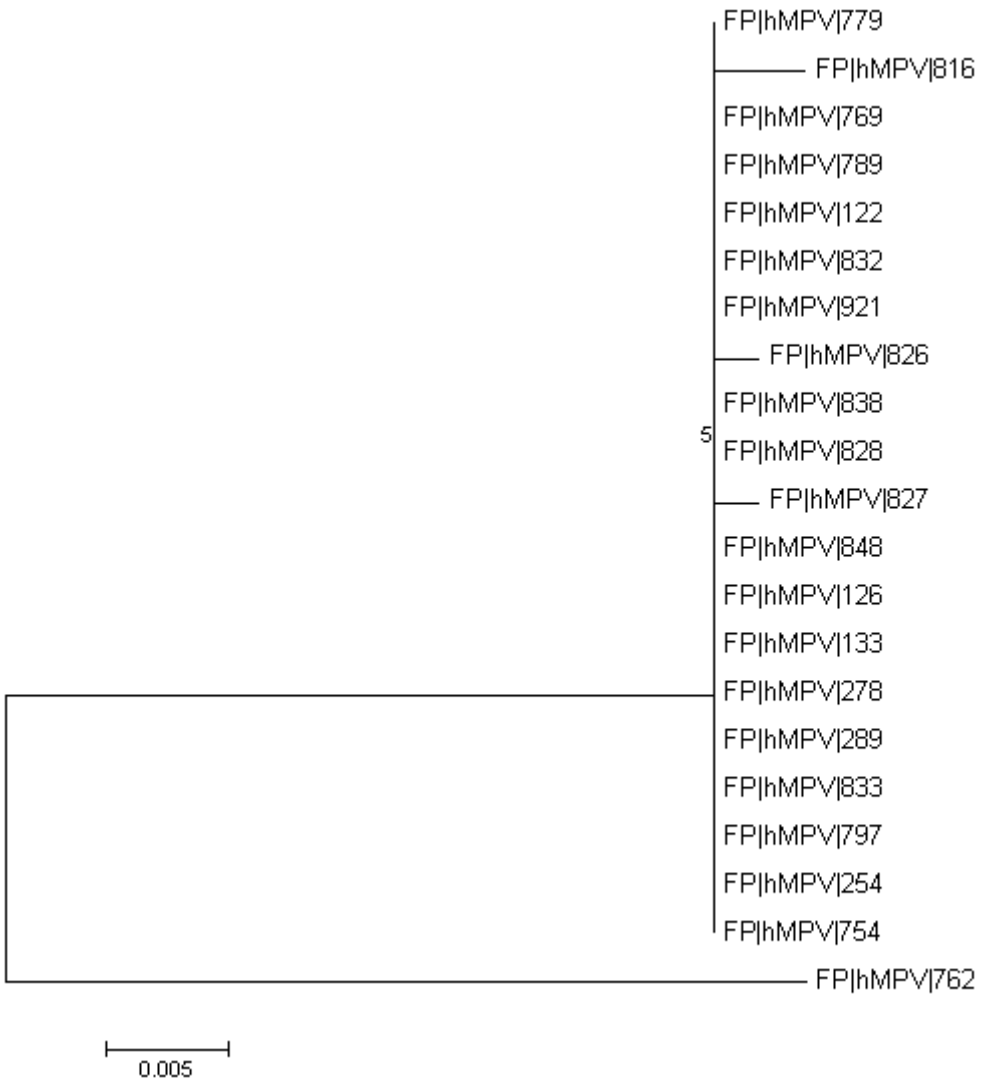


Figure 5.3. Phylogenetic analysis of partial F gene of hMPV strains. Analysis using a part of the F genes (537 nt) was performed for 21 hMPV strains collected from an Indonesian birth cohort between February 1999 to May 2001. A phylogenetic tree was constructed using the neighbour-joining method with 1,000 pseudoreplicate datasets. hMPV strains isolated are designated by an abbreviation FP|hMPV| followed by their unique episode number.

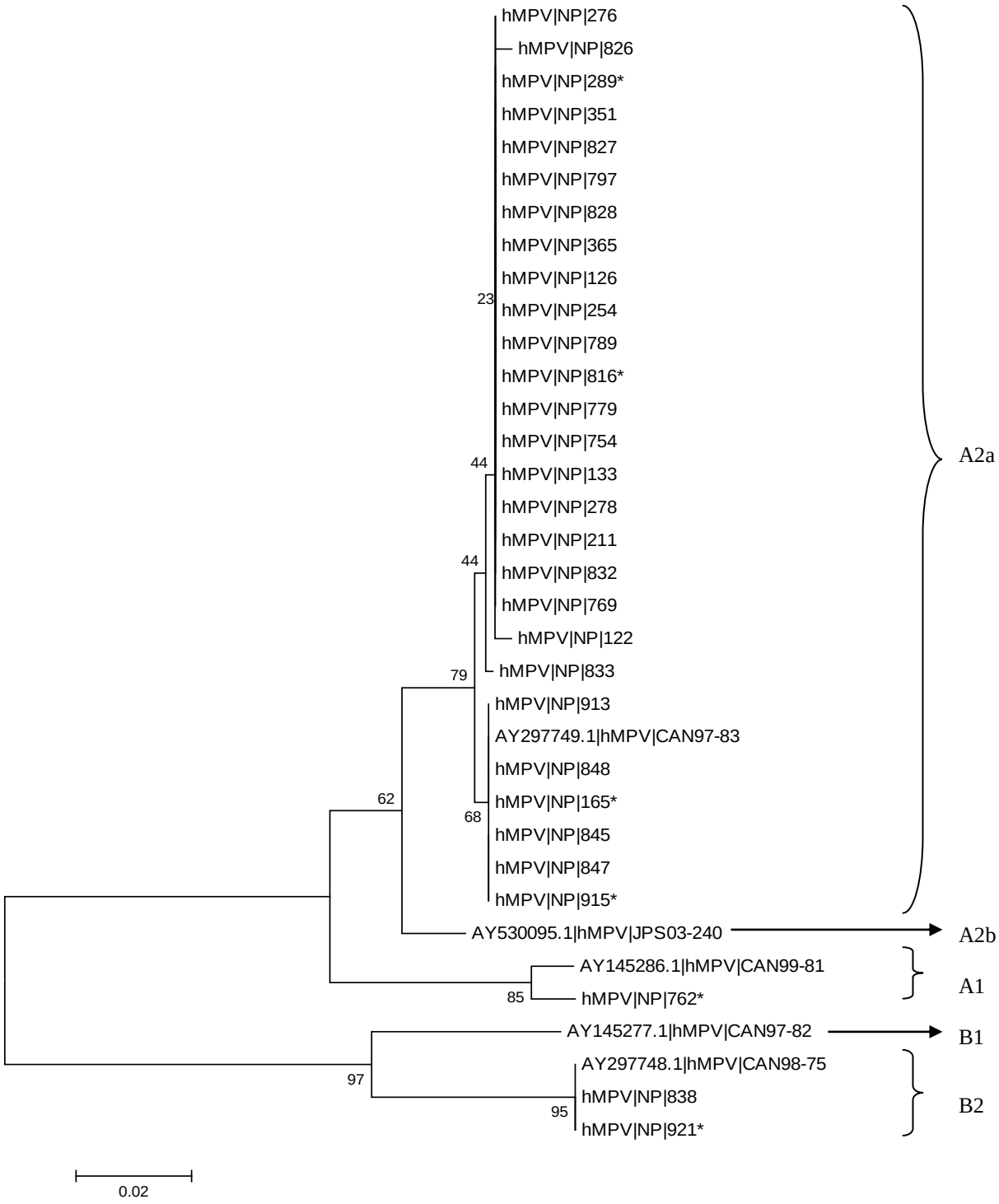

hMPV|NP|276
hMPV|NP|826
hMPV|NP|289*
hMPV|NP|351
hMPV|NP|827
hMPV|NP|797
hMPV|NP|828
hMPV|NP|365
hMPV|NP|126
23
hMPV|NP|254
hMPV|NP|789
hMPV|NP|816*
hMPV|NP|779
hMPV|NP|754
44
hMPV|NP|133
hMPV|NP|278
hMPV|NP|211
44
hMPV|NP|832
hMPV|NP|769
hMPV|NP|122
hMPV|NP|833
79
hMPV|NP|913
AY297749.1|hMPV|CAN97-83
hMPV|NP|848
62
hMPV|NP|165*
68
hMPV|NP|845
hMPV|NP|847
hMPV|NP|915*
AY530095.1|hMPV|JPS03-240
AY145286.1|hMPV|CAN99-81
85
hMPV|NP|762*
AY145277.1|hMPV|CAN97-82
97
AY297748.1|hMPV|CAN98-75
hMPV|NP|838
95
hMPV|NP|921*
0.02
A2a
A2b
A1
B1
B2

Figure 5.4. Phylogenetic analysis of partial N gene of hMPV strains. Analysis using a part of the N genes (350 nt) was performed for 30 hMPV strains collected from an Indonesian birth cohort between February 1999 to May 2001. A phylogenetic tree was constructed using the neighbour-joining method with 1,000 pseudoreplicate datasets. hMPV strains isolated are designated by an abbreviation hMPV|NP| followed by their unique episode number and * indicate severe pneumonia. The nomenclature is based on phylogenetic clustering with sequences previously assigned to specific genotypes obtained in Genbank (NCBI) designated by an abbreviation AY followed by their unique Genbank number.

Analysis of all 30 strains tested revealed that they belonged to three genotypes: 27 (90.0%) hMPV to genotype A2a, two (6.7%) to genotype B2 and one (3.3%) to genotype A1 (Figure 5.4). Based on figure 5.5, low incidence of hMPV genotype A2a has been circulating in this Indonesian population since January 1999 or before. However, a sudden increase in the number of hMPV cases detected in the first and second quarter of 2001 indicated that a small community outbreak might have occurred during that period. Upon analysis, the predominant strain remained as genotype A2a. However, the introduction of two relatively undetected genotypes for this cohort in the 28 months of collection: B2 and A1, were noted in the first quarter of 2001. This might signify the possibility of a switch in the predominant circulating strain for this population or the appearance and disappearance of these two genotype-specific viruses could just be another random event.

Comparing F gene sequences with the N gene sequences obtained for the 21 isolates in figures 5.3 and 5.4, it was noted that F gene sequences were unable to differentiate isolates 838 and 921 as B2 strains and placing them instead in the cluster of A2a strain. In this case, the N gene, though genetically more conserved than the F gene in the literature, was better able to differentiate the strains than F gene sequences.

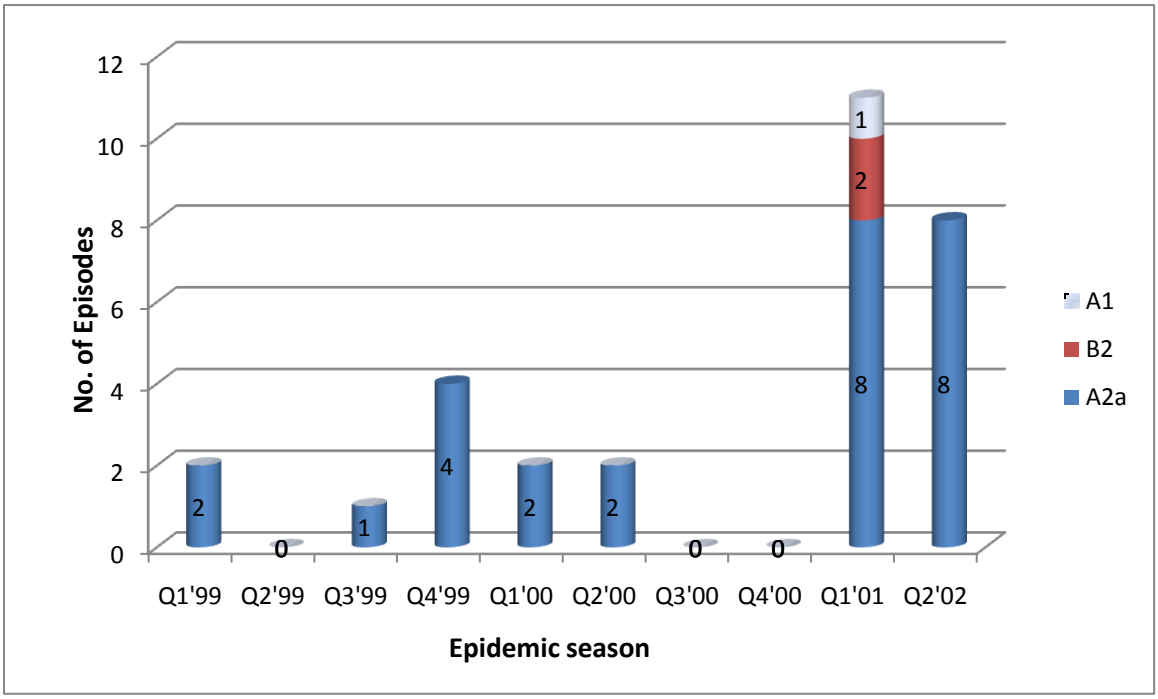


Figure 5.5. Seasonal frequencies of hMPV strains: A1, A2a and B2 strains collected during a 28 month period. All 30 strains of hMPV strains were used for the study of genetic diversity. Low incidence of hMPV genotype A2a was found to be circulating since first quarter of 1999. A sudden increase in numbers detected in the first and second quarters of 2001 indicated that a small community outbreak might have occurred. Q1' denotes January to March, Q2' denotes April to June, Q3' denotes July to September and Q4' denotes October to December. 99 denotes 1999, 00 denotes 2000 and 01 denotes 2001.

Out of the 30 strains analysed, six episodes of infections were associated with severe pneumonia: 289, 816, 915, 165, 762 and 921 (Figure 5.4). One episode each from the fourth quarter of 1999, first quarter of 2000 and second quarter of 2002 had severe pneumonia whereas three episodes of infections during the first quarter of 2001 were associated with severe pneumonia. However, an attempt to correlate a specific and predominant strain in patients with severe pneumonia was again unsuccessful as all three genotypes, i.e. A2a, A1 and B2 were identified in the six episodes of infections (Figure 5.5).

5.4 Discussion

5.4.1 Genetic Variability of RSV A

Variability between RSV strains is one of the features of RSV infections that might contribute to the ability of the virus to infect people repeatedly and cause yearly outbreaks. RSV has a clear seasonality with temperate climates having outbreaks occurring yearly in late fall, winter or spring but not in the summer. Both RSV groups can be present in the same community, and their relative proportions may differ between epidemics, although group A viruses tend to predominant (Viegas *et al.*, 2005; Parveen *et al.*, 2006; Reiche *et al.*, 2009).

In the present study, consistent annual peaks of RSV A from late January to June were observed over a 28 month period (February 1999 until May 2001). RSV from both subgroups were co-circulating during the 28 month period investigated in Indonesia; however RSV A strains were isolated more frequently and were predominant during the period. As for RSV B, only 12 isolates as compared to 73 isolates of RSV A were detected for the period. This finding was comparable with those of many other studies where higher prevalence was noted for RSV A than RSV B in many of the outbreaks (Frabasile *et al.*, 2003; Zlateva *et al.*, 2007; Reiche *et al.*, 2009). Previous

studies have reported results that indicate that RSV A and RSV B viruses alternate in prevalence during successive years (Sullender , 2000; Frabasile *et al.*, 2003), although such a finding was not observed from data in the present study. It has been speculated that the global prevalence of RSV A is due to the higher degree of divergence among viruses from this antigenic group. However, it is also possible that subgroup B may elicit a more complete and/or long-lasting subgroup-specific immune response than subgroup A and thus may cause illness less often (Zlateva *et al.*, 2007).

RSV group A is classified into several genotypes: GA1 to GA7 and SAA (Frabasile *et al.*, 2003; Reiche *et al.*, 2009). Three out of the eight genotypes circulated in Indonesia during the 28 month study period with more than one genotype detected within each season. Predominant genotypes observed in the present study were GA5, followed by GA2 and GA7. The finding of GA2 and GA5 as the most common genotypes of RSV A is in agreement with the results of long-term studies from Europe and other geographic areas. In these countries, mainly genotypes GA2 and GA5, and in a few cases, genotype GA7 have been circulating since 1998 (Frabasile *et al.*, 2003; Sato *et al.*, 2005; Viegas *et al.*, 2005; Parveen *et al.*, 2006; Reiche *et al.*, 2009). The continuous and predominant circulation of GA2 and GA5 demonstrated that these genotypes are stable and have become epidemic in many countries. A study conducted by Reiche *et al.*, (2009) suggested that either positive selection pressure or modulation of glycosylation sites among genotypes could have

contributed to the prolonged circulation of genotypes GA2 and GA5 in Germany. This could also be the case for our study cohort.

5.4.2 Genetic Variability of hMPV

This study presents for the first time findings reporting the presence of hMPV in community-acquired pneumonia patients in Indonesia. To better understand the epidemiology of this virus, genetic variation and the circulation pattern of hMPV was analysed over a 28 month period by sequence analysis of the nucleoprotein gene.

This study shows the molecular epidemiology and genetic variability of the hMPV circulating in Indonesia during a 28 month period. In previous studies, two groups of hMPV co-circulated and different subgroups dominate from year to year (Agapov *et al.*, 2006; Mackay *et al.*, 2006), which is similar to the circulation pattern of RSV (Chung *et al.*, 2008). Although four subtypes of hMPV co-circulated each year in an Italian study and an Australian study (Gerna *et al.*, 2005; Mackay *et al.*, 2006), other studies reported that a maximum of three subtypes circulated in any 1-year (Ludewick *et al.*, 2005; Chung *et al.*, 2008), which is consistent with the findings of this study, where the existence of three subgroups: A1, A2a and B2 were identified with A2a being the predominant strain. Interestingly, it was noted that the predominant

strains in Asian countries, such as Singapore, India and South Korea were of lineage A, in particular the sublineage A2 (Banerjee *et al.*, 2007; Chung *et al.*, 2007; Loo *et al.*, 2007). Whereas, lineage B tends to predominate in the Northern hemispheres, for examples: Italy and Croatia (Larcher *et al.*, 2005; Ljubin-Sternak *et al.*, 2008). However, in many other regions, co-circulations of both lineages with no predominance was also observed (Ludewick *et al.*, 2005; Matsuzaki *et al.*, 2008).

Phylogenetic analyses of strains of hMPV isolated in the present study reveal that the epidemiology of hMPV is complex and dynamic. Unlike influenza virus, where two or three strains spread across the globe each year, outbreaks of hMPV appear to be a local phenomenon, which explain why we tend to see genotype A more than genotype B in these regions of the world. Strains of hMPV differ from community to community, and strains identified in one location may be quite similar to strains identified in other locations in different years (Kahn, 2006). For example, the prototype strain identified in The Netherlands is genetically similar to strains identified in Australia and Quebec, Canada in different years (Kahn, 2006). Based on F gene sequences, viruses isolated in Australia in 2001, France in 2000 and 2002, Canada in 1999, 2000, 2001 and 2002, Israel in 2002 and The Netherlands in 2001 were closely related, with few polymorphisms in the F gene (Boivin *et al.*, 2004; Kahn, 2006). In any given year, viruses of both genotypes and both subgroups in each genotype) can circulate (Boivin *et al.,* 2004; Agapov *et al.,* 2006;

Kahn, 2006) but interestingly several studies had reported switching of predominant serotypes in consecutive years. Research by Agapov *et al.,* (2006) demonstrated a switch of the predominant genotype A to genotype B, while Ludewick *et al.,* (2005) noted a switch from genotype B2 to genotype A1. In South Korea, Chung *et al.,* (2008) noted that predominance of genotype A2a and A2b changed during their study period. For our study, predominance of genotypes A2a and a few cases of genotype A1 were noted in the early period, although subsequent detections of genotype B2 in the later part of the study was observed. This could signify the possibility of a switch in the predominant circulating strain for this population, fostered by a high prevalence of pre-existing community immunity to subgroup A, or it could mean a random event where genotype-specific viruses appeared and disappeared. The observed epidemiological features of hMPV are similar to those of RSV, where viruses of both A and B groups co-circulate each year and the predominant genotypes vary from location to location and from year to year (Alto *et al.*, 2004; van den Hoogen *et al.*, 2004; Camps *et al.*, 2008).

5.4.3 Molecular Epidemiology and Disease Severity

The clinical impact of viral genotypes and the relation between the disease severities are yet to be clarified. The current study attempted to correlate genotypes of hMPV and RSV A to determine if specific subgroups were

responsible for severe pneumonia observed in the cohorts investigated. RSV is divided into subgroups A and B, and some studies have reported that subgroup A isolates are associated with more severe illness (Walsh *et al.*, 1997; Gilca *et al.*, 2006), whereas others have found no differences between RSV subgroups (Fodha *et al.*, 2007; Kaplan *et al.*, 2008). This was also observed for hMPV, where controversial results are reported for hMPV genotypes A and B (Agapov *et al.*, 2006; Vicente *et al.*, 2006; Matsuzaki *et al.*, 2008). Our work could not detect any disease severity with any particular strain and these finding was supported by Agapov *et al.* (2006) where disease severity was not associated with any particular virus genotype. However, one limitation of the current study, and probably many others, is the relatively small numbers of patients with RSV A and hMPV, thus true differences in clinical severity may not be demonstrated. For RSV A, could the degradation of the 52 samples (out of the 73 samples) also be due to some strains being more prone to degradation in storage while others are more stable? If so, this could potential lead to bias of the results as correlation between certain viral strains and disease severity could have been established. In addition, if the community attack rate for a specific genotype for example RSV A viruses, was greater than group B viruses, which was often the case, a large number of severely ill patients might develop severe diseases during the group A-dominant period, even if the distribution of illness severity was identical for each virus subgroup (Fodha *et al.*, 2007). Thus, discrepancies could be attributed to differences in study design and populations, definitions of

disease severity and distribution shift from year to year (Gilca *et al.*, 2006). It is believed that additional studies on a larger population over several seasons are needed to better define the roles of hMPV and RSV genotypes in their pathogenesis. Nevertheless, the current study presented results showing that predominant strains isolated in RSV A: GA2, GA5 and GA7 were similar to those found in many other countries and predominant hMPV strains were A2a isolated mainly in the Asian countries (Parveen *et al.*, 2006; Banerjeer *et al.*, 2007; Chung *et al.*, 2007; Loo *et al.*, 2007; Reiche *et al.*, 2009). Though no novel lineages are observed in the present study, these findings are still important, especially for the development of new vaccines. The absence of novel lineages would probably mean that researcher can concentration on the development of vaccines for the current strains without the worries of its ineffectiveness on novel strains.

CHAPTER 6 – GENERAL DISCUSSION

In respiratory tract infections of humans, the relationship between host factors, virus strain, viral load and illness severity are often not well understood and poorly defined. The work presented in this thesis attempted to investigate viral factors (viral load, mixed infections, seasonality and viral strains) of hMPV, RSV and HRV contributing to disease severity in paediatric patients with pneumonia. The population studied was a paediatric cohort from Indonesia where nasopharyngeal washings were collected. This was a longitudinal study where infants and children enrolled were followed for 28 months (February 1999 to May 2001) and nasopharyngeal samples from both mild and severe pneumonia were collected. It was hypothesized that specific viral infections, increased viral loads, and the presence of specific viral strains as well as viral co-infections could result in more severe pneumonia in these infected individuals.

The approach to test the above hypothesis was to use an array-based pathogen detection method. An oligonucleotide array using Nimblegen array synthesis technology that was originally created by the Microarray team at the Genome Institute of Technology in Singapore was used in this study. A subset (approximately 10%) of the 352 nasopharyngeal samples from paediatric patients in this study cohort suffering mild and severe pneumonia, was screened initially for the presence of 35 RNA viruses using this microarray. Respiratory syncytial virus (RSV), human metapneumovirus (hMPV) and human rhinoviruses (HRVs) were detected in the 36 samples

tested. Taqman® real-time RT-PCR then was used to confirm the presence of these viruses in the samples and to determine the relative viral loads in each of the 36 samples prior to testing the rest of the nasopharyngeal samples. In addition to the RSV and hMPV pathogens, RNA viruses that commonly are associated with pneumonia in children include the influenza, parainfluenza and human enteroviruses (Sinaniotis *et al.*, 2004; Calvo *et al.*, 2008). The latter three viruses were included in the list of viruses detected by the pathogen detection platform designed in this study. It is interesting to note that none of these three viruses were detected in any of the 36 nasopharyngeal samples screened with the platform, inferring that the presence of influenza, parainfluenza and enteroviruses probably caused less than 10% of the pneumonia seen in this cohort, which was also observed by Calvo and colleagues (2008) in hospitalized infants.

For molecular epidemiological investigations of both RSV A and hMPV pathogens in the nasopharyngeal samples, sequencing of G protein was performed for 21of the 73 RSVA strains isolated and N protein was sequenced for all 30 strains of hMPV for phylogenetic analyses .

The epidemiology of major respiratory viruses causing infections in the paediatric population generally is similar in infected individuals from the developed countries, including Australia, Europe and the USA (Burgner *et al.*, 2005). RSV is the most important viral pathogen of infants and pre-school

children and is the predominant cause of viral lower respiratory tract infections in these populations (Burgner *et al.*, 2005; Viera *et al.*, 2007; Calvo *et al.*, 2008). Since its discovery in 2001, hMPV is now recognized as a substantial cause of acute respiratory infections of children and is found in most continents (Sloots *et al.*, 2006; Bosis *et al.*, 2008). However, this thesis outlines results of the first study that has detected the presence of hMPV in a paediatric population in Indonesia. The illness associated with hMPV infection clinically resembles that of RSV and ranges from mild upper; to severe lower respiratory tract disease (Sloots *et al.*, 2006; Manoha *et al.*, 2007). HRVs are the most common causes of viral illnesses worldwide and infections occur throughout the year in the human populations (Turner, 2007; Mackay *et al.*, 2008). Although once thought to cause only the common cold, the role of HRVs in lower respiratory infections and exacerbations of asthma conditions in infected adults and children has recently been appreciated (Miller *et al.*, 2007; Turner, 2007; Mackay *et al.*, 2008). For the current study, HRVs were found to be the most common viruses detected in the pneumonia cohort, in agreement with similar findings reported by other groups investigating acute respiratory tract infections (ARTIs) of children (Arden *et al.*, 2006; Miller *et al.*, 2007). An article by Turner (2007) queried the potential role of HRV infections as a cause of pneumonia, the results of the current study however indicate that the risk of pneumonia due to HRVs is similar to that caused by RSV A and hMPV.

Many studies conducted to further investigate the etiologies of ARTIs have published results obtained using nasopharyngeal samples from hospitalized patients as these patients usually were suffering from severe diseases (Arden *et al.*, 2006; Miller *et al.*, 2007; Calvo *et al.*, 2008) and the afore-mentioned studies were investigating the possible etiologies of the severe diseases seen in these patients. One of the strengths of this present study is the cohort of patients enrolled for the investigation. This longitudinal study not only allowed an investigation into both mild and severe pneumonia, it also enabled tracking of respiratory re-infections in paediatric patients. Hospital studies also usually lack control populations (i.e. patients with mild non-hospitalized disease) in their studies. Thus, our current work had an added advantaged as patients with mild pneumonia were incorporated as a control group for the study. In theory, viruses should be detected more commonly in hospitalized, rather than in non-hospitalized, infected individuals as a cause of severe illness and RSV that has been reported in many studies to be a common cause of severe infection (Fodha *et al.*, 2007; Okiro *et al.*, 2008). However, in this present study, RSV (RSV A and RSV B), hMPV and HRVs were found to have similar prevalence in both mild and severe pneumonia in the paediatric cohort studied. One inference that could be made from these findings is that specific viral infections of the respiratory tract in this paediatric population are not associated with the severity of disease seen in this population, and that the outcome of disease in this case is likely to be attributed to factors beyond simply the viral infection itself. However, one

aspect to note is that the frequencies of these viruses in healthy individuals with no disease symptoms has yet to be established and patients who were asymptomatic carriers were also not investigated. These factors warrant careful investigations as the detection of viruses in respiratory samples may not imply that they are true pathogens causing infections in patients.

The use of viral load to determine the severity of respiratory tract disease has been studied in various populations of infected children (Fodha *et al.*, 2007; Gerna *et al.*, 2007; Martin *et al.*, 2008). However, the relationship between the quantity of viral nucleic acids in respiratory secretions of infected individuals and the severity of illness attributed to respiratory viruses remains unclear. The quantity and quality of the nasopharyneal samples collected and used in these studies could be one factor that contributes to these discrepancies (Lambert *et al.*, 2008). Thus, for a more accurate method to assess the viral copy numbers in non-homogenous nasopharyngeal samples, a standardization process was developed using human 18S rRNA to normalize for the number of cells collected. This calculation method using viral copy number to the number of cells collected was a relative method used to normalize the nasopharngeal samples for better sample comparison. In 2004, when this present study was begun, it was amongst one of the first to investigate the association of disease severity with viral load. In addition, it was also one of the first studies attempting to normalise viral load per µL of nasopharyngeal sample to patient cells

collected to correct for variation in the collection process. Prior to normalization of samples in this study, slight increases in viral load were noted for HRV, RSV A and RSV B in samples taken from patients with severe pneumonia. However, after normalisation of samples, it was found that increased viral copy numbers (RSV A, RSV B, hMPV and HRV) did not result in an increase in severe disease among those affected individuals. It could be possible that severe disease led to more cellular damage which resulted in more cells being dislodged during sample collections or simply due to inflammatory response which lead to more fluids being produced and in turn lead to cell-dilutions. Thus, when normalised with the number of cells present in the nasopharyngeal washes, the median viral load of patients with both mild and severe pneumonia are statistically non-significant for all viruses (RSV A, RSV B, hMPV and HRV) tested. Lastly, one most important point to note is that no matter how good a sample has been collected from the upper respiratory tract (i.e. nasopharyngeal samples), it can and will always be only an indicative of the viral load in the lung tissues.

Studies into the clinical aspects of disease associated with viral infections of the lower respiratory tract have provided limited information and conflicting results (Devincenzo, 2004; Oliveira *et al.*, 2008). However, it has become apparent that it is important to understand the possible differences in disease severity caused by different subtypes and strains of viruses causing such infections because more therapeutic and prophylactic modalities against

viruses like RSV have become available (Nokes *et al.*, 2008; Olszewska *et al.*, 2009). An understanding of such differences in disease severity may have an effect on treatment strategies employed for the infected individuals (Papadoulos *et al.*, 2004; Oliveira *et al.*, 2008). Results from this current study show that RSV A predominated over RSVB as the causative agent of infections in the cohort studied although RSV subtype did not correlate with the severity of illness in these patients. This is in accordance with results of studies by DeVincenzo *et al.*, (2004) and Fodha *et al.*, (2007) who reported that the RSV subtype was not correlated significantly with the severity of illness. However, there is a limitation of the results presented in our current study concerning statistical power. Due to the relatively small number of patients with both RSVA and RSV B infections, differences in disease severity associated with these viral subtypes cannot be statistically demonstrated in the present study. In a similar scenario to that seen with RSV subtypes and severity of illness, there is conflicting evidence in the literature on the correlation of severity of disease caused by infection of the respiratory tract with various hMPV genotypes (Broor *et al.*, 2008; Carr *et al.*, 2008). In a very large study by Agapov *et al.*, (2006), no difference in the severity of respiratory tract disease caused by infection of this site with different hMPV genotypes were identified while Vicente *et al.* (2006) noted hMPV genotype A was more frequently isolated in cases of severe disease . As with RSV, our findings show no evidence of severe pneumonia associated with any specific hMPV genotypes. Carr *et al.*, (2008) suggested that these

discrepancies may be due to genetic variability and immunogenicity of hMPV, allowing for a seasonal shift in predominant circulating genotypes and the maintenance of the infection rate. For HRV, due to the complexity of their 100 over serotypes and the lack of time, we were unfortunately unable to proceed with the sequencing study. However, in the context of disease severity, it is important to sequence these positives HRV samples in the near future, especially since recent studies had identified a novel HRV species, HRV-C and associating it with severe pneumonia (Lau *et al*., 2009; Piralla *et al*., 2009). As HRV is the most prevalent pathogen of the 3 viruses detected in our pneumonia cases, it would therefore be of importance to also know if HRV-C is significantly associated with more severe pneumonia in our pediatric population.

One major result of investigations in this current study was the observation of mixed viral infections in cases of severe pneumonia. A significant ($P = 0.006$) association of RSV A and HRV co-infections with severe disease was found in the population that was studied. It was also noted that ***not all mixed infections lead to severe pneumonia but rather that specific combinations of co-infections had a significant effect on the outcome of the disease.*** Synergistic impact of the combination of these two viruses remains unclear, but, it is possible that each infection occurred in series rather than in parallel. It may also be possible for one infection to prime the host for subsequent infections perhaps by temporarily exhausting the innate immune response as

suggested by Arden *et al.,* (2006). Though sequencing and genotyping of the HRV strain associated with co-infection and disease severity was not done in this study, it is possible that this particular strain of HRV could be the notoriously pathogenic newly discovered HRV C strain associated with expiratory wheezing and pneumonia (McErlean *et al.*, 2007; Mackay *et al.*, 2008). Nevertheless, the observation that co-infections with HRV and RSV A causes severe pneumonia is a powerful piece of information as it could determine the management of patients with respiratory tract infections when the laboratory findings indicate the presence of both HRV and RSV A. Aggressive, timely and proper therapeutic measures should also be provided for these paediatric patients who are in the high risk group to prevent severe development of the disease.

This study has shown that HRV, RSV and hMPV can cause both mild and severe respiratory tract diseases in infected hosts and it has also shown that viral loads and viral strains observed could not explain the differences in clinical outcome. However, an intriguing finding was that co-infection with RSV A and HRV was associated with more severe illness in the cohort of patients studied. The initial microarray analysis that led to the investigation of these viruses was only undertaken on 36 samples and only looked for 35 viruses. It would be intriguing to further extend these studies, using a larger cohort set, a microarray chip with the ability to detect more viruses in patient samples as this may lead to the discovery of more co-infections that may

possibly account for more severe disease states seen in the infected individuals. In addition, multiple comparisons including host and environmental factors; which are known risk factors for severe disease should also be studied. It is known that in this Indonesian population, over 70% of the families have smoking members in the household, and the average family size being 6 is significantly larger than family sizes in Europe and North America (Eric Simoes, personal communication). Perhaps in the future study, a different population should also be included for comparative analysis with this current Indonesian population.

Results of the present study have led to a better understanding of the viruses including RSV A, RSV B, hMPV and HRV that contribute to disease severity in acute respiratory infections. Much more research is required to fully understand the various aspects of why some patients infected with these viruses are more susceptible than others to severe disease. It has been proven in this study that screening nasopharyngeal samples of infected individuals through array-based technology can be useful in determining the present of possible pathogens causing respiratory infections. A potential area for future investigation will be the detection of more viruses in the one sample and this will be achievable following finalization of the design of a pathogen detection chip that can detect the presence of up to 960 virus clusters probed on the array. This will enable screening nasopharyngeal aspirates for viruses, and thus increase viral detection rates in these infected individuals. However,

the need to improve sensitivities and to bring down the cost for routine clinical use of the arrays remains the most challenging.

In addition to viral factors, risk factors related to host and environment are also important indicators as to the outcome of respiratory infections. For RSV, the risk of severe infection is increased by premature birth, low birth weight (or "small for dates"), male gender, large family size, exposure to passive smoke, lack of breast feeding and by birth approximately 4 months before the peak of the RSV season (Rossi *et al.*, 2007; Olszewska *et al.*, 2009). In recent years, studies investigating host immune responses, and genetic susceptibilities to infectious diseases have been increasingly undertaken (Burgner *et al.*, 2006; Kelly *et al.*, 2008). It was noted by Kelly and colleagues that viral respiratory infections can have a profound effect on many aspects of asthma including its inception, exacerbations, and possibly, severity of disease (Kelly *et al.*, 2008). Thus, disease severity is likely to be determined by interplay between host and virus factors and investigations in both these aspects will most likely shed more light on the causes which may contribute to patients developing severe diseases.

APPENDICES

Appendix 1: List of the 35 viruses detected by the pathogen detection platform.

Genome	NCBI no.	Ref type	Accession no.	Description
1	9629198	RefSeq	NC_001781.1	Human respiratory syncytial virus, complete genome
2	19718363	RefSeq	NC_003461.1	Human parainfluenza virus 1 strain Washington/1964, complete genome
3	19525721	RefSeq	NC_003443.1	Human parainfluenza virus 2, complete genome
4	10937870	RefSeq	NC_001796.2	Human parainfluenza virus 3, complete genome
5	30468042	Genbank	AY283794.1	SARS coronavirus Sin2500, complete genome
6	38018022	RefSeq	NC_005147.1	Human coronavirus OC43, complete genome
7	12175745	RefSeq	NC_002645.1	Human coronavirus 229E, complete genome
8	46852132	RefSeq	NC_004148.2	Human metapneumovirus, complete genome
9	8486138	RefSeq	NC_002023.1	Influenza A virus RNA segment 1, complete sequence
	8486136	RefSeq	NC_002022.1	Influenza A virus RNA segment 3, complete sequence
	8486134	RefSeq	NC_002021.1	Influenza A virus RNA segment 2, complete sequence
	8486131	RefSeq	NC_002020.1	Influenza A virus RNA segment 8, complete sequence
	8486129	RefSeq	NC_002019.1	Influenza A virus RNA segment 5, complete sequence
	8486127	RefSeq	NC_002018.1	Influenza A virus RNA segment 6, complete sequence
	8486125	RefSeq	NC_002017.1	Influenza A virus RNA segment 4, complete sequence
10	8486164	RefSeq	NC_002204.1	Influenza B virus RNA-1, complete sequence
	8486148	RefSeq	NC_002205.1	Influenza B virus RNA-2, complete sequence
	8486150	RefSeq	NC_002206.1	Influenza B virus RNA-3, complete sequence
	8486152	RefSeq	NC_002207.1	Influenza B virus RNA-4, complete sequence
	8486154	RefSeq	NC_002208.1	Influenza B virus RNA-5, complete sequence
	8486156	RefSeq	NC_002209.1	Influenza B virus RNA-6, complete sequence
	8486159	RefSeq	NC_002210.1	Influenza B virus RNA-7, complete sequence
	8486161	RefSeq	NC_002211.1	Influenza B virus RNA-8, complete sequence
11	11528013	RefSeq	NC_001563.2	West Nile virus, complete genome
12	9627244	RefSeq	NC_002031.1	Yellow fever virus, complete genome
13	13559808	RefSeq	NC_002728.1	Nipah virus, complete genome

Genome	NCBI no.	Ref type	Accession no.	Description
14	11545722	RefSeq	NC_002617.1	Newcastle disease virus, complete genome
15	9629357	RefSeq	NC_001802.1	Human immunodeficiency virus 1, complete genome
16	21326584	RefSeq	NC_003977.1	Hepatitis B virus, complete genome
17	9627257	RefSeq	NC_001576.1	Human papillomavirus type 10, complete genome
18	10445391	RefSeq	NC_002554.1	Foot-and-mouth disease virus C, complete genome
19	9790308	RefSeq	NC_001545.1	Rubella virus, complete genome
20	9626732	RefSeq	NC_001489.1	Hepatitis A virus, complete genome
21	38371716	RefSeq	NC_005222.1	Hantaan virus, complete genome
22	38371727	RefSeq	NC_005217.1	Sin Nombre virus, complete genome
23	23334588	RefSeq	NC_004294.1	Lymphocytic choriomeningitis virus segment S, complete sequence
	23334585	RefSeq	NC_004291.1	Lymphocytic choriomeningitis virus segment L, complete sequence
24	9626460	RefSeq	NC_001437.1	Japanese encephalitis virus, genome
25	51850386	DNA Data	AB189128.1	Dengue virus type 3 genomic RNA, complete genome, strain: 98902890 DF DV-3
26	12659201	Genbank	AF326573.1	Dengue virus type 4 strain 814669, complete genome
27	19744844	Genbank	AF489932.1	Dengue Virus Type 2 strain BR64022, complete genome
28	323660	Genbank	M87512.1	DENT1SEQ Dengue virus type 1 complete genome
29	9626436	RefSeq	NC_001430.1	Human enterovirus D, complete genome
30	9626433	RefSeq	NC_001428.1	Human enterovirus C, complete genome
31	9627719	RefSeq	NC_001612.1	Human enterovirus A, complete genome
32	21363125	RefSeq	NC_003986.1	Human echovirus 1, complete genome
33	9626677	RefSeq	NC_001472.1	Human enterovirus B, complete genome
34	9627730	RefSeq	NC_001617.1	Human rhinovirus 89, complete genome
35	9626735	RefSeq	NC_001490.1	Human rhinovirus B, complete genome

Appendix 2: Pathogen Chip Sample Amplification and Array Hybridization Protocol (Adapted from Wong *et al.*, 2007)

Pathogen Chip Sample Amplification and Array Hybridization Protocol

Primer A2: 5' GATGAGGGAAGATGGGGNNNNNNNN
Primer B2: 5' GATGAGGGAAGATGGGG

Round A: 1st strand synthesis with RT

Mix 2 μl RNA (1:10) with 2 μl primer "A2" (20 μM stock) to a final volume of 10 μl
Heat to 65 °C /5 mins
Cool at room temperature (25 °C)/5 mins
Add 10 μl of 2× enzyme mix

2× enzyme mix:
- 2.0 μl 10× RT Buffer (Stratagene)
- 1.0 μl 10 mM dNTP mix (final concentration 500 μM each nucleotide)
- 3.0 μl H_2O
- 2.0 μl 0.1 mM DTT
- 2.0 μl Stratascript Reverse Transcriptase (Stratagene)

Incubate at 42 °C/30 mins
Heat to 65 °C /5 mins
Cool at room temperature (25 °C)/5 mins
Add 1 μl RT
Incubate additional 42 °C /30 mins
Hold at 4 °C

2nd strand synthesis with Sequenase

Heat sample to 94 °C /2 mins
Rapidly cool to 10 °C, hold at 10 °C /5 mins
Add 10 μl Sequenase mix for a total RXN volume of 30 μl

Sequenase Mix:
- 2.0 μl 5× Sequenase Buffer
- 7.7 μl H_2O
- 0.3 μl Sequenase (USB)

Ramp from 10 °C to 37°C over 8 mins, hold at 37 °C /8 mins
Rapid ramp to 94 °C, hold at 94 °C /2 mins
Rapid ramp to 10 °C , hold at 10 °C /5 mins while adding 1.2 μl of diluted Sequenase (1:4 dilution)
Ramp from 10 °C to 37 °C over 8 mins, hold at 37 °C/8mins
Hold at room temperature
Sample total volume = 30 μl

Round B: PCR amplification

Round A Template	30 µl
10× PCR Buffer	10
50 mM $MgCl_2$	4
10 mM dNTP	2.5
Primer B2 (100 µM stock)	1
Platinum *Taq* (Invitrogen)	1
ddH_2O	51.5

Round B cycles:
Ramp to 94 °C C, hold at 94 °C C/8 mins
94 °C C/30 secs
40 °C C/30 secs } 40×
50 °C C/30 secs
72 °C C/1 min
4 °C C/∞
Each PCR product total volume = 100 µl
Loaded 5 µl into 1% agarose gel before proceed on to concentration (to 6 µl).

Digestion of Sample:

Make up 0.1U/ µl DNase I:

Stock 5U/ µl DNase I	2 µl
ddH_2O	98 µl

Make up Digestion mix:

10× One-Phor-All Buffer	1 µl	} good for 1 array
0.1 U/µl DNase I	1 µl	
ddH_2O	2 µl	
TOTAL	4 µl	

For each sample:

Round B DNA	6 µl
Digestion mix	4 µl

Incubate at 37 °C /3 mins
Incubate at 97 °C /15 mins; spin down each 5 mins or so
Place on ice for at least 3 mins

Biotin labeling:

Digested product	10 µl
Biotin-N6-ddATP	1 µl
Terminal transferase	2 µl

Incubate at 37 °C /90 mins (can start prehyb after 1 hr)
Incubate at 97 °C /15 mins; spin down each 5 mins or so

Preparation of prehybridization and hybridization solutions:

For each microarray being hybridized, prepare the following 2 solutions in separate 1.5 ml microcentrifuge tubes.
Note: If sample volume is less than 13 µl, top-up with ddH_2O.

Component	Prehyb solution (µl)	Hyb solution (µl)
Fragmented PCR products	--	13
CPK6 Oligo (100 nM)	--	0.45
Herring sperm DNA (10 mg/ml)	--	1
2× TMAC Resequencing buffer (Nimblegen proprietary)	100	16
5 M TMAC	--	6
ddH_2O	100	--
TOTAL	200	36.45

Prehybridization:

1. Transfer prehybridization solution to 95 °C heat block/5 mins
2. Incubate at 45 °C/5 mins (at MAUI station)
3. Spin at 13000 rpm/5 mins (check for precipitation)
4. Pipet 200 µl of prehybridization solution to the array. Avoid forming bubbles. Place hybrislip cover
5. Incubate slide in MAUI chamber at 45 °C/ 20 mins
6. During this incubation, the hybridization solution can be prepared for use as described in the next section.
7. Remove hybri-strip from slide while immersed in ddH_2O
8. Wash in ddH_2O/1 min
9. Wash in 70% ethanol/1 min
10. Dry by centrifuging at 100 ×g/4 mins

Hybridization:

1. Transfer hybridization solution to 95 °C heat block/5 mins
2. Incubate at 45 °C/5 mins (at MAUI station)
3. Spin at 13000 rpm/5 mins (check for precipitation)
4. Transfer to 45 °C heat block until prehybridization step has been completed
5. Adhere MAUI cover to slide and place inside MAUI hyb station
6. Spin briefly to collect sample and pipette 36.45 µl of sample into one of the two MAUI cover holes
7. Dry any sample leakage around the holes
8. Adhere MAUI stickers to both holes
9. Click down the fastener and start hybridization (program A)
10. Hybridize overnight (~16.5 hrs: 5.30pm- 10.00am)

Washes and Staining:

Prepare all washes, stains and antibody amplification mixes prior to removing chip from MAUI

Wash 1 (dunk bath) – 2× SSC/0.1% SDS
Wash 2 – 0.2× SSC
Wash 3 – 0.05× SSC
Wash 4 – 70% ethanol
2× Stain buffer – put in 42 °C water bath 15 mins prior stain preparation

1× Stain solution (100 mM MES, 1M [Na^+], 0.05% Tween-20):

2× Stain buffer	1500 µl
ddH_2O (non DEPC-treated)	1410 µl
Non-acetylated BSA (100 mg/ml)	60 µl
Cy3-Streptavidin (1 µg/µl)	30 µl
TOTAL	3000 µl

1× Antibody amplification solution:

2× Stain buffer	1500 µl
Non-acetylated BSA (100 mg/ml)	96 µl
Goat IgG (50 mg/ml)	8.64 µl
Biotinylated Goat anti-SA (0.5 mg/ml)	28.8 µl
ddH_2O (non DEPC-treated)	1367 µl
TOTAL	3000 µl

Dunk stain solution:

2× Stain buffer	22.5 ml
ddH_2O (non DEPC-treated)	22.5 ml
1× Stain solution	3 ml
TOTAL	48 ml

Dunk antibody amplification solution:

2× Stain buffer	22.5 ml
ddH_2O (non DEPC-treated)	22.5 ml
1× Antibody amplification solution	3 ml
TOTAL	48 ml

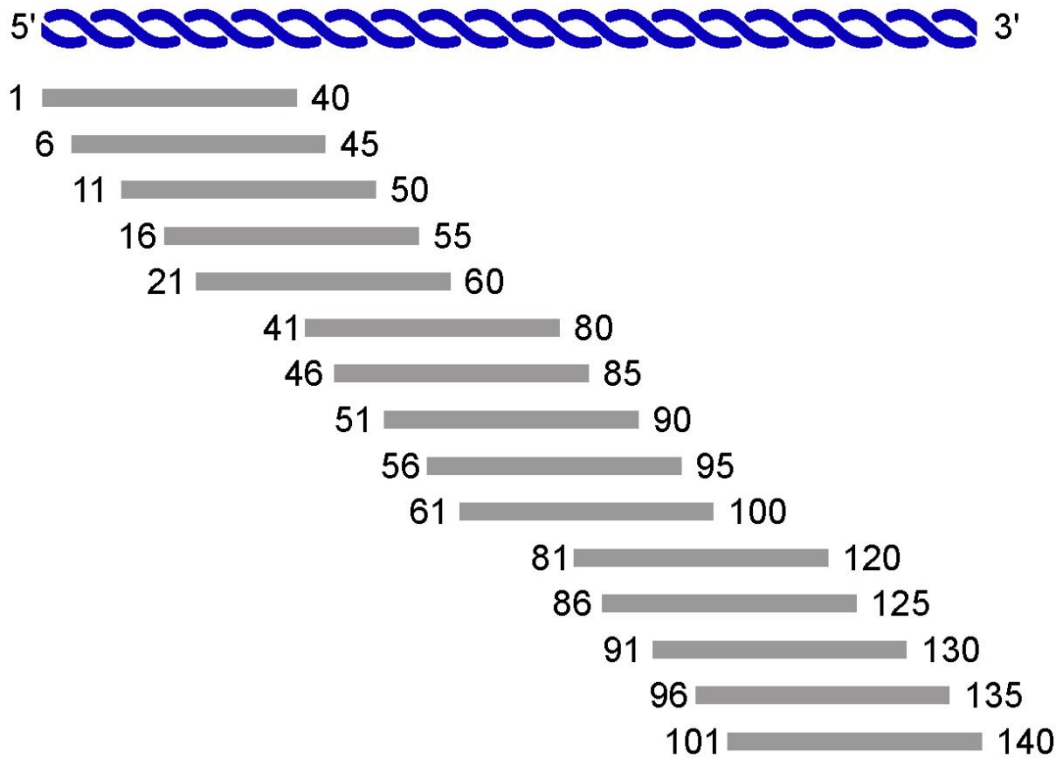
* Preparation of Goat IgG (50 mg/ml)
1 ml 150 mM NaCl + 50 mg of Goat IgG

Preparation of 150 mM NaCl
225 µl 1 M NaCl + 1275 µl ddH_2O

1. Stop MAUI, open fastener, remove chip with MAUI lid still attached.
2. Remove MAUI lid from array while immersed in Wash 1. Using 2 hands crack the cover off the slide using slide edge.
3. Transfer to Wash 2 for 1 min

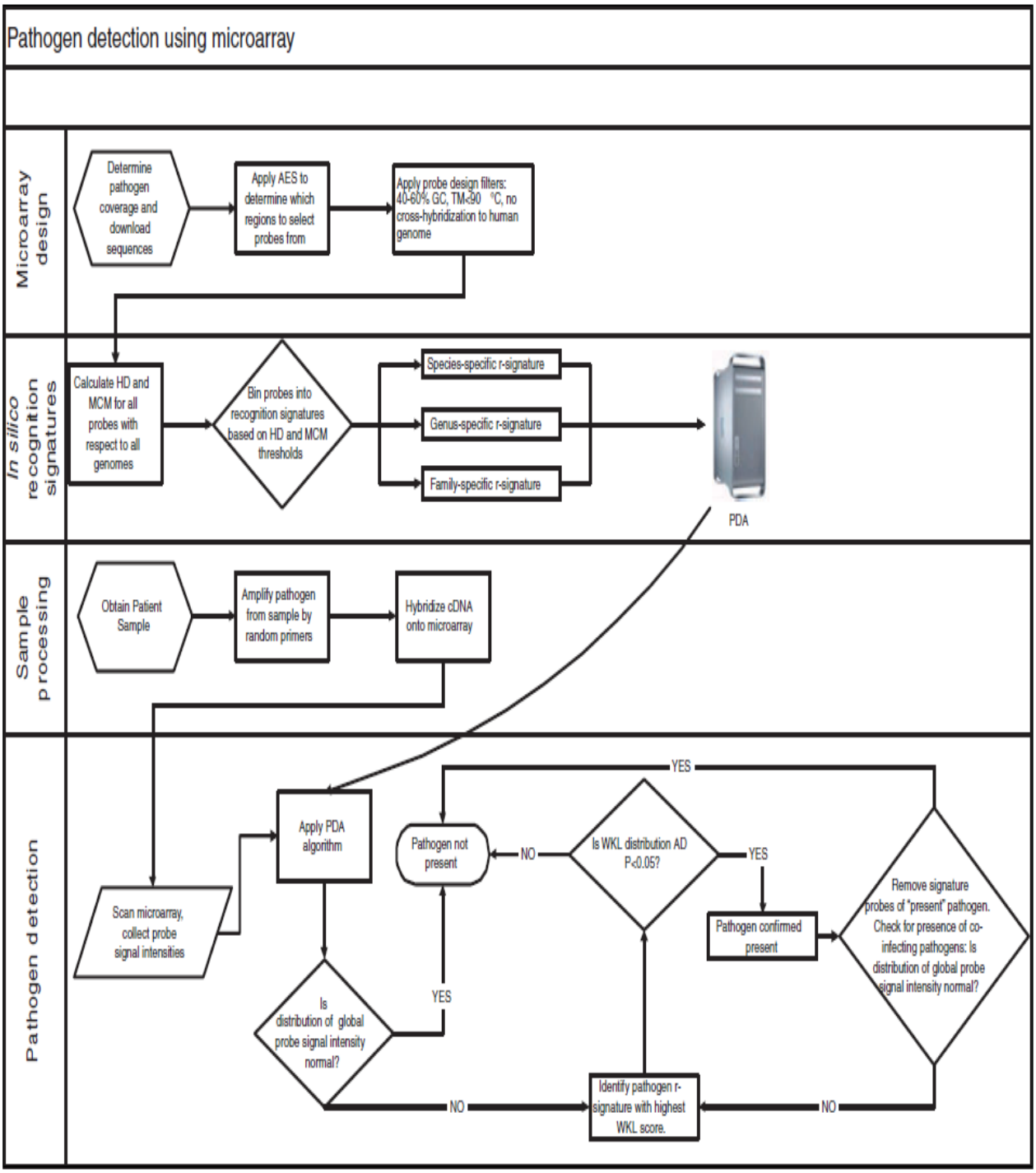
4. Remove each slide from Wash 2. Place into stain solution (3 arrays/coplin jar of 48 ml solution). Let stand in dark for 10 mins. Invert jar to mix 3× over 10 mins.
5. Handling one slide at a time, transfer each slide back into Wash 2 for a quick wash (reuse from step 3)/1 min.
6. Place into antibody amplification solution (3 arrays/coplin jar of 48 ml solution).
7. Let stand in dark for 10 mins. Invert jar to mix 3× over 10 mins.
8. Handling one slide at a time, transfer each slide back into Wash 2 for a quick wash (reuse from step 3)/1 min.
9. Place into stain solution (3 arrays/coplin jar of 48 ml solution). Let stand in dark for 10 mins. Invert jar to mix 3× over 10 mins.
10. Transfer the slides to Wash 2/ 1 mins (new wash)
11. Transfer to Wash 3/ 30".
12. Dunk 6× in 70% ethanol.
13. Dry array by centrifuging 100 ×g/4 mins
14. Scan array on Axon scanner (within 6 hrs of wash) at 400 PMT, 5 μm resolution, no averaging (using GenePix Pro 4 software)

Appendix 3: Probe design schema.



This illustrates the tiling probes created across the genome of NC_001781 Human respiratory syncytial virus (RSV). Probes covering the first 140 nucleotides are shown. The numbers represent the start and end positions of each probe. 1948 probes were synthesized to cover the entire 15225 bp RSV genome. This process was repeated for the remaining 34 viral genomes.

Appendix 4: Schema of pathogen detection process (Adapted from Wong *et al.*, 2007)



Appendix 5. Primers trialled for real-time RT-PCR detection of hMPV

Primer Set[a]	Probe (nM)[b]	Concentration of control plasmid (per uL)									
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}
T1a	**200**	20.9	24.0	28.0	30.8	34.9	38.9	U[c]	U	U	U
	300	20.8	24.5	27.7	30.5	34.0	U	U	U	U	U
	400	20.9	24.6	27.7	31.1	34.1	U	U	U	U	U
T1b	**200**	22.9	26.3	29.5	32.7	36.0	U	U	U	U	U
	300	22.6	25.8	29.4	32.8	36.2	U	U	U	U	U
	400	22.4	25.9	29.0	32.4	35.6	U	U	U	U	U
T2a	**200**	20.7	24.0	27.1	30.4	34.0	U	U	U	U	U
	300	20.9	24.2	27.6	30.5	33.6	U	U	U	U	U
	400	21.0	24.5	27.7	30.4	34.9	U	U	U	U	U
T2b	**200**	22.8	26.0	29.3	32.6	35.5	U	U	U	U	U
	300	22.7	25.9	29.3	32.5	36.4	U	U	U	U	U
	400	22.5	25.8	29.1	32.6	35.8	U	U	U	U	U
T3a[d]	**200**	21.4	24.7	27.9	31.3	34.6	40.0	U	U	U	U
	300	21.5	24.8	28.3	31.6	34.4	U	U	U	U	U
	400	21.6	25.1	28.1	31.8	34.4	38.9	U	U	U	U
T3b	**200**	23.2	26.7	30.0	33.4	38.1	U	U	U	U	U
	300	23.3	26.5	29.8	33.5	37.5	U	U	U	U	U
	400	22.7	26.7	29.9	33.5	38.0	U	U	U	U	U
T4a	**200**	21.9	25.0	28.7	32.2	36.5	U	U	U	U	U
	300	22.1	25.6	28.9	32.1	35.6	U	U	U	U	U
	400	22.6	25.7	29.3	32.5	38.2	U	U	U	U	U
T4b	**200**	24.6	28.0	31.2	34.6	38.7	U	U	U	U	U
	300	25.0	28.3	31.7	35.5	U	U	U	U	U	U
	400	26.1	29.0	32.3	36.3	U	U	U	U	U	U

[a]denotes primers and probes combination as below:
T1a = F1FT + F1RT + F1PT (FAM/TAMRA)
T1b = F1FT + F1RT + F2PT (HEX/TAMRA)
T2a = F1FT + F2RT + F1PT (FAM/TAMRA)
T2b = F1FT + F2RT + F2PT (HEX/TAMRA)
T3a = N1FT + N1RT + N1PT (FAM/TAMRA)
T3b = N1FT + N1RT + N2PT (HEX/TAMRA)
T4a = N2FT + N2RT + N1PT (FAM/TAMRA)
T4b = N2FT + N2RT + N2PT (HEX/TAMRA)
[b] denotes concentration of probes in nM
[c] denotes undetermined by real-time RT-PCR
[d] denotes primers and probe sets used for the detection of hMPV in this study

Appendix 6: Primers used for sequencing of RSV A

Reaction	Primer name	Sequence (5'-3')
Reverse transcription	RSVArtR	CAGGAAACAGCTATGACACGAGAA AAAAAGTGTCAAAAACT
1st PCR reaction	RSVAglycoF1	GTAAAACGACGGCCAGTGAATATAA CGTATTCCATAACAA
	RSVAglycoR1	CAGGAAACAGCTATGACACTAAGATA GCCTTTGCTAACT
2nd PCR reaction	RSVAshortF	AGYATATGCAGCAACAATCCA
	RSVAshortR	GCAAHTCCATTGTTATTTGCC
1st Sequencing reaction	RSVAseqf	GTAAAACGACGGCCAG
	RSVAseqr	CAGGAAACAGCTATGAC
	RSVAglycoF2	ATACTAGCTTBAACAACACCA
	RSVAglycoR2	AGGGTACAAAGTTGAACACTT
2nd Sequencing reaction	RSVAshortF	AGYATATGCAGCAACAATCCA
	RSVAshortR	GCAAHTCCATTGTTATTTGCC

REFERENCES

AGAPOV, E., SUMINO, K., GAUDREAULT-KEENER, M., STORCH, G. & HOLTZMAN, M. 2006. Genetic variability of human metapneumovirus infection: evidence of a shift in viral genotype without a change in illness. *J Infect Dis,* 193**,** 396-403.

AHLUWALIA, G. & HAMMOND, G. 1988. Comparison of cell culture and three enzyme-linked immunosorbent assays for the rapid diagnosis of respiratory syncytial virus from nasopharyngeal aspirate and tracheal secretion specimens. *Diagn Microbiol Infect Dis,* 9**,** 187-92.

ALLANDER, T., ANDREASSON, K., GUPTA, S., BJERKNER, A., BOGDANOVIC, G., PERSSON, M., DALIANIS, T., RAMQVIST, T. & ANDERSSON, B. 2007. Identification of a third human polyomavirus. *J Virol,* 81**,** 4130-6.

ALLANDER, T., TAMMI, M., ERIKSSON, M., BJERKNER, A., TIVELJUNG-LINDELL, A. & ANDERSSON, B. 2005. Cloning of a human parvovirus by molecular screening of respiratory tract samples. *Proc Natl Acad Sci U S A,* 102**,** 12891-6.

ALTO, W. Human metapneumovirus: a newly described respiratory tract pathogen. 2004. *J Am Board Fam Pract,* 17**,** 466-9.

ANEVLAVIS, S., PETROGLOU, N., TZAVARAS, A., MATLEZOS, S., PNEUMATIKOS, I., FROUDARAKIS, M., ANEVLAVIS, E. & BOUROS, D. 2009. A prospective study of the diagnostic utility of sputum Gram stain in pneumonia. *J of Infect,* 59, 83-89.

ARABPOUR, M., SAMARBAFZADEH, A., MAKVANDI, M., SHAMSIZADEH, A., PERCIVALLE, E., ENGLUD, J. & LATIFI, S. 2008. The highest prevalence of human metapneumovirus in Ahwaz children accompanied by acute respiratory infections. *Indian J Med Microbiol,* 26**,** 123-6.

ARDEN, K. & MACKAY, I. 2009. Human rhinoviruses: coming in from the cold. *Genome Med,* I:44.

ARDEN, K., MCERLEAN, P., NISSEN, M., SLOOTS, T. & MACKAY, I. 2006. Frequent detection of human rhinoviruses, paramyxoviruses, coronaviruses, and bocavirus during acute respiratory tract infections. *J Med Virol,* 78**,** 1232-40.

ARNOLD, J., SINGH, K., SPECTOR, A. & SAWYER, M. 2006. Human bocavirus: prevalence and clinical spectrum at a children's hospital. *Clin Infect Dis,* 43, 283-8.

ATKINSON, M., YANNEY, M., STEPHENSON, T. & SMYTH, A. 2007. Effective treatment strategies for paediatric community-acquired pneumonia. *Expert Opin Pharmacother,* 8**,** 1091-101.

BANERJEE, S., BHARAJ, P., SULLENDER, W., KABRA, S. & BROOR, S. 2007. Human metapneumovirus infections among children with acute respiratory infections seen in a large referral hospital in India. *J Clin Virol,* 38**,** 70-2.

BANNISTER, C., RUSSELL, S., RIMMER, S., THORNE, J. & HELLINGS, S. 2000. Can prognostic indicators be identified in a fetus with an encephalocele? *Eur J Pediatr Surg,* 10 Suppl 1**,** 20-3.

BARBEN, J., KUEHNI, C., TRACHSEL, D. & HAMMER, J. 2008. Management of acute bronchiolitis: can evidence based guidelines alter clinical practice? *Thorax,* 63**,** 1103-9.

BAS, A., FORSBERG, G., HAMMARSTRÖM, S. & HAMMARSTRÖM, M. 2004. Utility of the housekeeping genes 18S rRNA, beta-actin and glyceraldehyde-3-phosphate-dehydrogenase for normalization in real-time quantitative reverse transcriptase-polymerase chain reaction analysis of gene expression in human T lymphocytes. *Scand J Immunol,* 59**,** 566-73.

BASTIEN, N., LIU, L., WARD, D., TAYLOR, T. & LI, Y. 2004. Genetic variability of the G glycoprotein gene of human metapneumovirus. *J Clin Microbiol,* 42**,** 3532-7.

BELLAU-PUJOL, S., VABRET, A., LEGRAND, L., DINA, J., GOUARIN, S., PETITJEAN-LECHERBONNIER, J., POZZETTO, B., GINEVRA, C. & FREYMUTH, F. 2005. Development of three multiplex RT-PCR assays for the detection of 12 respiratory RNA viruses. *J Virol Methods,* 126**,** 53-63.

BIALASIEWICZ, S., WHILEY, D., LAMBERT, S., JACOB, K., BLETCHLY, C., WANG, D., NISSEN, M., & SLOOTS, T. 2008. Presence of the newly discovered human polyomaviruses KI and WU in Australian patients with acute respiratory tract infection. *J Clin Virol.* 2, 63-8.

BLOMQVIST S, SAVOLAINEN C, RÅMAN L, ROIVAINEN M, HOVI T. Human rhinovirus 87 and enterovirus 68 represent a unique serotype with rhinovirus and enterovirus features. 2002. *J Clin Microbiol.* 11, 4218-23.

BODROSSY, L. & SESSITSCH, A. 2004. Oligonucleotide microarrays in microbial diagnostics. *Curr Opin Microbiol,* 7**,** 245-54.

BOIVIN, G., ABED, Y., PELLETIER, G., RUEL, L., MOISAN, D., CÔTÉ, S., PERET, T., ERDMAN, D. & ANDERSON, L. 2002. Virological features and clinical manifestations associated with human metapneumovirus: a new paramyxovirus responsible for acute respiratory-tract infections in all age groups. *J Infect Dis,* 186**,** 1330-4.

BOIVIN, G., DE, S., COTE, S., GILCA, R., ABED, Y., & ROCHETTE, L. 2003. Human metapneumovirus infections in hospitalized children. *Emerg Infect Dis,* 9, 634-640.

BOIVIN, G., MACKAY, I., SLOOTS, T., MADHI, S., FREYMUTH, F., WOLF, D. & ET. AL. 2004. Global genetic diversity of human metapneumovirus fusion gene. *Emerg Infect Dis,* 10, 1154-7.

BORG, I., ROHDE, G., LÖSEKE, S., BITTSCHEIDT, J., SCHULTZE-WERNINGHAUS, G., STEPHAN, V. & BUFE, A. 2003. Evaluation of a quantitative real-time PCR for the detection of respiratory syncytial virus in pulmonary diseases. *Eur Respir J,* 21**,** 944-51.

BOSIS, S., ESPOSITO, S., OSTERHAUS, A., TREMOLATI, E., BEGLIATTI,

E., TAGLIABUE, C., CORTI, F., PRINCIPI, N. & NIESTERS, H. 2008. Association between high nasopharyngeal viral load and disease severity in children with human metapneumovirus infection. *J Clin Virol,* 42**,** 286-90.

BRITTAIN-LONG, R., NORD, S., OLOFSSON, S., WESTIN, J., ANDERSON, L. & LINDH, M. 2008. Multiplex real-time PCR for detection of respiratory tract infections. *J Clin Virol,* 41**,** 53-6.

BRIXNER, D. 2004. Clinical and economic outcomes in the treatment of lower respiratory tract infections. *Am J Manag Care,* 10**,** S400-7.

BROOKS, W., GOSWAMI, D., RAHMAN, M., NAHAR, K., FRY A., BALISH, A., IFEKHARUDDIN, N., AZIM, T., XU X., KLIMOV, A., BRESEE, J., BRIDGES, C. & LUBY, S. 2010. Influenza is a major contributor to childhood pneumonia in a tropical developing country. *PIDJ,* 29, 216-221.

BROOKS, B., LUTZE-WALLACE, C., LU, P. & ROBERTSON, R. 2004. Identification and serological specificity of a polysaccharide component from Mycoplasma bovis. *Vet Res Commun,* 28**,** 197-208.

BROOR, S., BHARAJ, P. & CHAHAR, H. 2008. Human metapneumovirus: a new respiratory pathogen. *J Biosci,* 33**,** 483-93.

BUCHHOLZ, U., BIACCHESI, S., PHAM, Q., TRAN, K., YANG, L., LUONGO, C., SKIADOPOULOS, M., MURPHY, B. & COLLINS, P. 2005. Deletion of M2 gene open reading frames 1 and 2 of human metapneumovirus: effects on RNA synthesis, attenuation, and immunogenicity. *J Virol,* 79**,** 6588-97.

BURGNER, D., JAMIESON, S. & BLACKWELL, J. 2006. Genetic susceptibility to infectious diseases: big is beautiful, but will bigger be even better? *Rev. Lancet Infect Dis,* 6, 653-663.

BURGNER, D. & RICHMOND, P. 2005. The burden of pneumonia in children: an Australian perspective. *Paediatr Respir Rev,* 6**,** 94-100.

CABEZUELO HUERTA, G., VIDAL MICÓ, S., ABELEDO GÓMEZ, A. & FRONTERA IZQUIERDO, P. 2005. [Underlying causes of recurrent pneumonia]. *An Pediatr (Barc),* 63**,** 409-12.

CALVO, C., GARCÍA-GARCÍA, M., BLANCO, C., VÁZQUEZ, M., FRÍAS, M., PÉREZ-BREÑA, P. & CASAS, I. 2008. Multiple simultaneous viral infections in infants with acute respiratory tract infections in Spain. *J Clin Virol,* 42**,** 268-72.

CAMPS, M., RICART, S., DIMOVA, V., ROVIRA, N., MUÑOZ-ALMAGRO, C., GARCIA, J., PONS-ODENA, M., MARCOS, M. & PUMAROLA, T. 2008. Prevalence of human metapneumovirus among hospitalized children younger than 1 year in Catalonia, Spain. *J Med Virol,* 80**,** 1452-60.

CAPEDING MR, SOMBRERO LT, PALADIN FJ, SUZUKI H, NUMAZAKI Y, SANIEL MC. 1994. Etiology of acute lower respiratory infection in Filipino children under five years. *Southeast Asian J Trop Med Public Health.* 25, 684-7.

CARR, M., WATERS, A., FENWICK, F., TOMS, G., HALL, W. & O'KELLY, E. 2008. Molecular epidemiology of human metapneumovirus in Ireland. *J Med Virol,* 80**,** 510-6.
CASABIANCA, A., ORLANDI, C., FRATERNALE, A. & MAGNANI, M. 2004. Development of a real-time PCR assay using SYBR Green I for provirus load quantification in a murine model of AIDS. *J Clin Microbiol,* 42**,** 4361-4.
CENTER FOR DISEASE CONTROL PREVENTION (CDC). 2003. Outbreak of severe acute respiratory syndrome - worldwide. *Morb Mortal Wkly Rep*. 52, 226-8.
CHAM, G., YAN, S., HENG, B. & SEOW, E. 2009. Predicting positive blood cultures in patients presenting with pneumonia at an Emergency Department in Singapore. *Ann Acad Med Singapore,* 38**,** 508-7.
CHAN, P., CHEW, F., TAN, T., CHUA, K. & HOOI, P. 2002. Seasonal variation in respiratory syncytial virus chest infection in the tropics. *Pediatr Pulmonol,* 34, 47-51.
CHAN, P., SUNG, R., FUNG, K., HUI, M., CHIK, K., ADEYEMI-DORO, F. & CHENG, A. 1999. Epidemiology of respiratory syncytial virus infection among paediatric patients in Hong Kong: seasonality and disease impact. *Epidemiol Infect,* 123**,** 257-62.
CHANO, F., ROUSSEAU, C., LAFERRIÈRE, C., COUILLARD, M. & CHAREST, H. 2005. Epidemiological survey of human metapneumovirus infection in a large pediatric tertiary care center. *J Clin Microbiol,* 43**,** 5520-5.
CHECCHIA, P. 2008. Identification and management of severe respiratory syncytial virus. *Am J Health Syst Pharm,* 65**,** S7-12.
CHERIAN, T., SIMOES, E., STEINHOFF, M., CHITRA, K., JOHN, M., RAGHUPATHY, P. & JOHN, T. 1990. Bronchiolitis in tropical south India. *Am J Dis Child,* 144**,** 1026-30.
CHEW, F., DORAISINGHAM, S., LING, A., KUMARASINGHE, G. & LEE, B. 1998. Seasonal trends of viral respiratory tract infections in the tropics. *Epidemiol Infect,* 121**,** 121-8.
CHIANG, W., TEOH, O., CHONG, C., GOH, A., TANG, J. & CHAY, O. 2007. Epidemiology, clinical characteristics and antimicrobial resistance patterns of community-acquired pneumonia in 1702 hospitalized children in Singapore. *Respirology,* 12**,** 254-61.
CHIEN, J. & JOHNSON, J. 2000. Viral pneumonias. Epidemic respiratory viruses. *Postgrad Med,* 107**,** 41-2, 45-7, 51-2.
CHUNG, J., HAN, T., KIM, S. & HWANG, E. 2008. Genotype variability of human metapneumovirus, South Korea. *J Med Virol,* 80**,** 902-5.
CIFTÇI, E., GÜNEŞ, M., KÖKSAL, Y., INCE, E. & DOĞRU, U. 2003. Underlying causes of recurrent pneumonia in Turkish children in a university hospital. *J Trop Pediatr,* 49**,** 212-5.
COCKERILL, M. 2005. BMC Bioinformatics comes of age. *BMC Bioinformatics,* 6**,** 140.

CONTOLI, M., MESSAGE, S., LAZA-STANCA, V., EDWARDS, M., WARK, P., BARTLETT, N., KEBADZE, T., MALLIA, P., STANCIU, L., PARKER, H., SLATER, L., LEWIS-ANTES, A., KON, O., HOLGATE, S., DAVIES, D., KOTENKO, S., PAPI, A. & JOHNSTON, S. 2006. Role of deficient type III interferon-lambda production in asthma exacerbations. *Nat Med,* 12**,** 1023-6.

COOPER, D., SMITH, G., EDMUNDS, W., JOSEPH, C., GERARD, E. & GEORGE, R. 2007. The contribution of respiratory pathogens to the seasonality of NHS Direct calls. *J Infect,* 55**,** 240-8.

COOTE, N. & MCKENZIE, S. 2000. Diagnosis and investigation of bacterial pneumonias. *Paediatr Respir Rev,* 1**,** 8-13.

CORSELLO, G., DI CARLO, P., SALSA, L., GABRIELE, B., MELI, L., BRUNO, S. & TITONE, L. Respiratory syncytial virus infection in a Sicilian pediatric population: risk factors, epidemiology, and severity. *Allergy Asthma Proc,* 29**,** 205-10.

CROWE, J. J. 2004. Human metapneumovirus as a major cause of human respiratory tract disease. *Pediatr Infect Dis J,* 23**,** S215-21.

CURRY, A., APPLETON, H. & DOWSETT, B. 2006. Application of transmission electron microscopy to the clinical study of viral and bacterial infections: present and future. *Micron,* 37**,** 91-106.

DEFFERNEZ, C., WUNDERLI, W., THOMAS, Y., YERLY, S., PERRIN, L. & KAISER, L. 2004. Amplicon sequencing and improved detection of human rhinovirus in respiratory samples. *J Clin Microbiol,* 42**,** 3212-8.

DEVINCENZO, J. 2004. Natural infection of infants with respiratory syncytial virus subgroups A and B: a study of frequency, disease severity, and viral load. *Pediatr Res,* 56**,** 914-7.

DEVINCENZO, J. 2007. A new direction in understanding the pathogenesis of respiratory syncytial virus bronchiolitis: how real infants suffer. *J Infect Dis,* 195**,** 1084-6.

DEVINCENZO, J. 2008. Passive antibody prophylaxis for RSV. *Pediatr Infect Dis J,* 27**,** 69-70.

DIMECH, W., BOWDEN, D., BRESTOVAC, B., BYRON, K., JAMES, G., JARDINE, D., SLOOTS, T. & DAX, E. 2004. Validation of assembled nucleic acid-based tests in diagnostic microbiology laboratories. *Pathology,* 36**,** 45-50.

DJELANTIK, I., GESSNER, B, SOEWIGNJO, S., STEINHOFF, M.,SUTANTO, A., WIDJAYA, A., LINEHAN, M., MONIAGA, V & INGERANI. 2003. Incidence and clinical features of hospitalization because of respiratory syncytial virus lower respiratory illness among children less than two years of age in a rural Asian setting. *Pediatr Infect Dis,* 22, 150-6.

DOWSON, C., JOHNSON, A., CERCENADO, E. & GEORGE, R. 1994. Genetics of oxacillin resistance in clinical isolates of Streptococcus pneumoniae that are oxacillin resistant and penicillin susceptible. *Antimicrob Agents Chemother,* 38**,** 49-53.

DRESCHERS, S., DUMITRU, C., ADAMS, C. & GULBINS, E. 2007. The cold case: are rhinoviruses perfectly adapted pathogens? *Cell Mol Life Sci,* 64**,** 181-91.

DRUMMOND, P., CLARK, J., WHEELER, J., GALLOWAY, A., FREEMAN, R. & CANT, A. 2000. Community acquired pneumonia--a prospective UK study. *Arch Dis Child,* 83**,** 408-12.

DUNCAN, A. 2005. Pneumonia in intensive care--another frontier? *Anaesth Intensive Care,* 33**,** 11-2.

EBIHARA, T., ENDO, R., KIKUTA, H., ISHIGURO, N., ISHIKO, H., HARA, M., TAKAHASHI, Y. & KOBAYASHI, K. 2004. Human metapneumovirus infection in Japanese children. *J Clin Microbiol,* 42**,** 126-32.

ESCOBAR, C., LUCHSINGER, V., DE OLIVEIRA, D., DURIGON, E., CHNAIDERMAN, J. & AVENDAÑO, L. 2009. Genetic variability of human metapneumovirus isolated from Chilean children, 2003-2004. *J Med Virol,* 81**,** 340-4.

EWIG, S., SCHLOCHTERMEIER, M. & GOKE, N. 2002. Applying sputum as a diagnostic tool in pneumonia: limited yield, minimal impact on treatment decisions. *Chest,* 121: 1486-92.

FARHA, T. & THOMSON, A. 2005. The burden of pneumonia in children in the developed world. *Paediatr Respir Rev,* 6**,** 76-82.

FELDMAN, C. 2004. Appropriate management of lower respiratory tract infections in primary care. *Prim Care Respir J,* 13**,** 159-66.

FELDMAN, R., RHEW, D., WONG, J., CHARLES, R. & GOETZ, M. 2003. Azithromycin monotherapy for patients hospitalized with community-acquired pneumonia: a 31/2-year experience from a veterans affairs hospital. *Arch Intern Med,* 163**,** 1718-26.

FERRONI, A., NGUYEN, L., GEHANNO, P., BOUCOT, I. & BERCHE, P. 1996. Clonal distribution of penicillin-resistant Streptococcus pneumoniae 23F in France. *J Clin Microbiol,* 34**,** 2707-12.

FIORE, A., SHAY, D., BRODER, K., ISKANDER, J., UYEKI, T., MOOTREY, G., BRESEE, J. & COX, N. 2009. Prevention and control of seasonal influenza with vaccines: recommendations of the Advisory Committee on Immunization Practices (ACIP), 2009. *MMWR Recomm Rep,* 58**,** 1-52.

FODHA, I., VABRET, A., GHEDIRA, L., SEBOUI, H., CHOUCHANE, S., DEWAR, J., GUEDDICHE, N., TRABELSI, A., BOUJAAFAR, N. & FREYMUTH, F. 2007. Respiratory syncytial virus infections in hospitalized infants: association between viral load, virus subgroup, and disease severity. *J Med Virol,* 79**,** 1951-8.

FRABASILE, S., DELFRARO, A., FACAL, L., VIDELA, C., GALIANO, M., DE SIERRA, M., RUCHANSKY, D., VITUREIRA, N., BEROIS, M., CARBALLAL, G., RUSSI, J. & ARBIZA, J. 2003. Antigenic and genetic variability of human respiratory syncytial viruses (group A)

isolated in Uruguay and Argentina: 1993-2001. *J Med Virol,* 71**,** 305-12.

FUCHS, S., FISCHER, G., BLACK, R. & LANATA, C. 2005. The burden of pneumonia in children in Latin America. *Paediatr Respir Rev,* 6**,** 83-7.

GASC, A., GESLIN, P. & SICARD, A. 1995. Relatedness of penicillin-resistant Streptococcus pneumoniae serogroup 9 strains from France and Spain. *Microbiology,* 141 (Pt 3)**,** 623-7.

GAYNOR, A., NISSEN, M., WHILEY, D., MACKAY, I., LAMBERT, S., WU, G., BRENNAN, D., STORCH, G., SLOOTS, T. & WANG, D. 2007. Identification of a novel polyomavirus from patients with acute respiratory tract infections. *PLoS Pathog,* 3**,** e64.

GERNA, G., CAMPANINI, G., ROGNONI, V., MARCHI, A., ROVIDA, F., PIRALLA, A. & PERCIVALLE, E. 2008. Correlation of viral load as determined by real-time RT-PCR and clinical characteristics of respiratory syncytial virus lower respiratory tract infections in early infancy. *J Clin Virol,* 41**,** 45-8.

GESTELAND, P., SAMORE, M., PAVIA, A., SRIVASTAVA, R., KORGENSKI, K., GERBER, K., DALY, J., MUNDORFF, M., ROLFS, R., JAMES, B. & BYINGTON, C. 2007. Informing the front line about common respiratory viral epidemics. *AMIA Annu Symp Proc***,** 274-8.

GILCA, R., DE SERRES, G., TREMBLAY, M., VACHON, M., LEBLANC, E., BERGERON, M., DERY, P. & BOIVIN, G. 2006. Distribution and clinical impact of human respiratory syncytial virus genotypes in hospitalized children over 2 winter seasons. *J Infect Dis,* 193**,** 54-8.

GILLIM-ROSS, L. & SUBBARAO, K. 2006. Emerging respiratory viruses: challenges and vaccine strategies. *Clin Microbiol Rev,* 19**,** 614-36.

GRAHAM, S., ENGLISH, M., HAZIR, T., ENARSON, P. & DUKE, T. 2008. Challenges to improving case management of childhood pneumonia at health facilities in resource-limited settings. *Bull World Health Organ,* 86**,** 349-55.

GREENBERG, S. 2003. Respiratory consequences of rhinovirus infection. *Arch Intern Med,* 163**,** 278-84.

GREENSILL, J., MCNAMARA, P., DOVE, W., FLANAGAN, B., SMYTH, R. & HART, C. 2003. Human metapneumovirus in severe respiratory syncytial virus bronchiolitis. *Emerg Infect Dis,* 9**,** 372-5.

GUEUDIN, M., VABRET, A., PETITJEAN, J., GOUARIN, S., BROUARD, J. & FREYMUTH, F. 2003. Quantitation of respiratory syncytial virus RNA in nasal aspirates of children by real-time RT-PCR assay. *J Virol Methods,* 109**,** 39-45.

HAMANO-HASEGAWA, K., MOROZUMI, M., NAKAYAMA, E., CHIBA, N., MURAYAMA, S., TAKAYANAGI, R., IWATA, S., SUNAKAWA, K. & UBUKATA, K. 2008. Comprehensive detection of causative pathogens using real-time PCR to diagnose pediatric community-acquired pneumonia. *J Infect Chemother,* 14**,** 424-32.

HAN, T., CHUNG, J., KOO, J., KIM, S., & HWANG, E. 2007. WU polyomavirus in children with acute lower respiratory tract infections, South Korea. *Emerg Infect Dis.* 13, 1766-8.

HAYDEN, F. 2004. Rhinovirus and the lower respiratory tract. *Rev. Med Virol,* 14, 17-31.

HENRICKSON, K. & HALL, C. 2007. Diagnostic assays for respiratory syncytial virus disease. *Pediatr Infect Dis J,* 26**,** S36-40.

HENRICKSON, K., HOOVER, S., KEHL, K. & HUA, W. 2004. National disease burden of respiratory viruses detected in children by polymerase chain reaction. *Pediatr Infect Dis J,* 23**,** S11-8.

HU, A., COLELLA, M., TAM, J., RAPPAPORT, R. & CHENG, S. 2003. Simultaneous detection, subgrouping, and quantitation of respiratory syncytial virus A and B by real-time PCR. *J Clin Microbiol,* 41**,** 149-54.

HUCK, B., SCHARF, G., NEUMANN-HAEFELIN, D., PUPPE, W., WEIGL, J. & FALCONE, V. 2006. Novel human metapneumovirus sublineage. *Emerg Infect Dis,* 12**,** 147-50.

HUERTA C, LANES SF, GARCÍA RODRÍGUEZ LA. 2005. Respiratory medications and the risk of cardiac arrhythmias. *Epidemiology.* 16, 360-6.

JARTTI, T., LEHTINEN, P., VUORINEN, T., KOSKENVUO, M. & RUUSKANEN, O. 2004. Persistence of rhinovirus and enterovirus RNA after acute respiratory illness in children. *J Med Virol,* 72**,** 695-9.

JUVÉN, T., MERTSOLA, J., WARIS, M., LEINONEN, M., MEURMAN, O., ROIVAINEN, M., ESKOLA, J., SAIKKU, P. & RUUSKANEN, O. 2000. Etiology of community-acquired pneumonia in 254 hospitalized children. *Pediatr Infect Dis J,* 19**,** 293-8.

KAFETZIS, D. 2004. Prophylaxis, therapy and prevention of viral respiratory infections. *Paediatr Respir Rev,* 5 Suppl A**,** S185-9.

KAHN, J. 2006. Epidemiology of human metapneumovirus. *Clin Microbiol Rev,* 19**,** 546-57.

KAIDA, A., KUBO, H., GOTO, K., SHIOMI, M., KOHDERA, U. & IRITANI, N. 2007. Co-infection of human metapneumovirus with adenovirus or respiratory syncytial virus among children in Japan. *Microbiol Immunol,* 51**,** 679-83.

KAPLAN, N., DOVE, W., ABD-ELDAYEM, S., ABU-ZEID, A., SHAMOON, H. & HART, C. 2008. Molecular epidemiology and disease severity of respiratory syncytial virus in relation to other potential pathogens in children hospitalized with acute respiratory infection in Jordan. *J Med Virol,* 80**,** 168-74.

KELLY, J. & BUSSE, W. 2008. Host immune responses to rhinovirus: mechanisms in asthma. *J Allergy Clin Immunol,* 122**,** 671-82; quiz 683-4.

KESSON, A. 2007. Respiratory virus infections. *Ped Res Rev,* 8: 240-248.

KIM, S., KI, C. & LEE, N. 2009. Rapid detection and identification of 12 respiratory viruses using a dual priming oligonucleotide system-based multiplex PCR assay. *J Virol Methods,* 156**,** 111-6.
KLEIN, M., COVIELLO, S., BAUER, G., BENITEZ, A., SERRA, M., SCHIATTI, M., DELGADO, M., MELENDI, G., NOVALLI, L., PENA, H., KARRON, R., KLEEBERGER, S. & POLACK, F. 2006. The impact of infection with human metapneumovirus and other respiratory viruses in young infants and children at high risk for severe pulmonary disease. *J Infect Dis,* 193**,** 1544-51.
KLINGSPOR, L. & LOEFFLER, J. 2009. Aspergillus PCR formidable challenges and progress. *Med Mycol,* 47 Suppl 1**,** S241-7.
KORPPI, M., HEISKANEN-KOSMA, T., LEINONEN, M. & HALONEN, P. 1993. Antigen and antibody assays in the aetiological diagnosis of respiratory infection in children. *Acta Paediatr,* 82**,** 137-41.
KORPPI, M., KOTANIEMI-SYRJÄNEN, A., WARIS, M., VAINIONPÄÄ, R. & REIJONEN, T. 2004. Rhinovirus-associated wheezing in infancy: comparison with respiratory syncytial virus bronchiolitis. *Pediatr Infect Dis J,* 23**,** 995-9.
KSIAZEK, T., ERDMAN, D., GOLDSMITH, C., ZAKI, S., PERET, T., EMERY, S., TONG, S., URBANI, C., COMER, J., LIM, W., ROLLIN, P., DOWELL, S., LING, A., HUMPHREY, C., SHIEH, W., GUARNER, J., PADDOCK, C., ROTA, P., FIELDS, B., DERISI, J., YANG, J., COX, N., HUGHES, J., LEDUC, J., BELLINI, W. & ANDERSON, L. 2003. A novel coronavirus associated with severe acute respiratory syndrome. *N Engl J Med,* 348**,** 1953-66.
KUMAR, S., TAMURA, K. & NEI, M. 2004. MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. *Brief Bioinform,* 5**,** 150-63.
KUROIWA, Y., NAGAI, K., OKITA, L., YUI, I., KASE, T., NAKAYAMA, T. & TSUTSUMI, H. 2005. A phylogenetic study of human respiratory syncytial viruses group A and B strains isolated in two cities in Japan from 1980-2002. *J Med Virol,* 76**,** 241-7.
KUYPERS, J., WRIGHT, N. & MORROW, R. 2004. Evaluation of quantitative and type-specific real-time RT-PCR assays for detection of respiratory syncytial virus in respiratory specimens from children. *J Clin Virol,* 31**,** 123-9.
KUYPERS, J., WRIGHT, N., COREY, L. & MORROW, R. 2005. Detection and quantification of human metapneumovirus in pediatric specimens by real-time RT-PCR. *J Clin Virol,* 33**,** 299-305.
KUYPERS, J., WRIGHT, N., FERRENBERG, J., HUANG, M., CENT, A., COREY, L. & MORROW, R. 2006. Comparison of real-time PCR assays with fluorescent-antibody assays for diagnosis of respiratory virus infections in children. *J Clin Microbiol,* 44**,** 2382-8.
LAMBERT, S., WHILEY, D., O'NEILL, N., ANDREWS, E., CANAVAN, F., BLETCHLY, C., SIEBERT, D., SLOOTS, T. & NISSEN, M. 2008.

Comparing nose-throat swabs and nasopharyngeal aspirates collected from children with symptoms for respiratory virus identification using real-time polymerase chain reaction. *Pediatrics,* 122**,** e615-20.

LANDRY, M., FERGUSON, D., COHEN, S., PERET, T. & ERDMAN, D. 2005. Detection of human metapneumovirus in clinical samples by immunofluorescence staining of shell vial centrifugation cultures prepared from three different cell lines. *J Clin Microbiol,* 43: 1950-1952.

LARCHER, C., PAGANI, E., ROSSI, P., AMATO, B., PESCOLLDERUNGG, L., CAMPANINI, G., PERCIVALLE, E. & HUEMER, H. 2008. Comparison of human metapneumovirus genotypes from the province of Bolzano in northern Italy with strains from surrounding regions in Italy and Austria. *Jpn J Infect Dis,* 61**,** 154-6.

LAU, S., YIP, C., TSOI, H., LEE, R., SO, L., LAU, Y., CHAN, K., WOO, P. & YUEN, K. 2007. Clinical features and complete genome characterization of a distinct human rhinovirus (HRV) genetic cluster, probably representing a previously undetected HRV species, HRV-C, associated with acute respiratory illness in children. *J Clin Microbiol,* 45**,** 3655-64.

LAU, S., YIP, C., WOO, P., YUEN, KY. 2009. Human rhinovirus C: a newly discovered human rhinovirus species. *Emerg Health Threats J,* 3:e2.

LEDFORD, R., COLLETT, M. & PEVEAR, D. 2005.Insights into the genetic basis for natural phenotypic resistance of human rhinoviruses to pleconaril. *Antiviral Res,* 68, 135-138.

LEE, J., CHANG, L., WANG, L., KAO, C., SHAO, P., LU, C., LEE, P., CHEN, J., LEE, C. & HUANG, L. 2007. Epidemiology of respiratory syncytial virus infection in northern Taiwan, 2001-2005 -- seasonality, clinical characteristics, and disease burden. *J Microbiol Immunol Infect,* 40**,** 293-301.

LEE, N., CHAN, P., HUI, D., RAINER, T., WONG, E., CHOI, K., LUI, G., WONG, B., WONG, R., LAM, W., CHU, I., LAI, R., COCKRAM, C. & SUNG, J. 2009. Viral loads and duration of viral shedding in adult patients hospitalized with influenza. *J Infect Dis,* 200**,** 492-500.

LEE, W., WONG, C., LEONG, W., MILLER, L. & SUNG, W. 2008. LOMA: a fast method to generate efficient tagged-random primers despite amplification bias of random PCR on pathogens. *BMC Bioinformatics,* 9**,** 368.

LEFMANN, M., HONISCH, C., BÖCKER, S., STORM, N., VON WINTZINGERODE, F., SCHLÖTELBURG, C., MOTER, A., VAN DEN BOOM, D. & GÖBEL, U. 2004. Novel mass spectrometry-based tool for genotypic identification of mycobacteria. *J Clin Microbiol,* 42**,** 339-46.

LEMANSKE, R. J., JACKSON, D., GANGNON, R., EVANS, M., LI, Z., SHULT, P., KIRK, C., REISDORF, E., ROBERG, K., ANDERSON, E., CARLSON-DAKES, K., ADLER, K., GILBERTSON-WHITE, S.,

PAPPAS, T., DASILVA, D., TISLER, C. & GERN, J. 2005. Rhinovirus illnesses during infancy predict subsequent childhood wheezing. *J Allergy Clin Immunol,* 116**,** 571-7.
LICHENSTEIN, R., SUGGS, A. & CAMPBELL, J. 2003. Pediatric pneumonia. *Emerg Med Clin North Am,* 21**,** 437-51.
LIN, B., WANG, Z., VORA, G., THORNTON, J., SCHNUR, J., THACH, D., BLANEY, K., LIGLER, A., MALANOSKI, A., SANTIAGO, J., WALTER, E., AGAN, B., METZGAR, D., SETO, D., DAUM, L., KRUZELOCK, R., ROWLEY, R., HANSON, E., TIBBETTS, C. & STENGER, D. 2006. Broad-spectrum respiratory tract pathogen identification using resequencing DNA microarrays. *Genome Res,* 16**,** 527-35.
LIU, J., LIM, S., RUAN, Y., LING, A., NG, L., DROSTEN, C., LIU, E., STANTON, L. & HIBBERD, M. 2005. SARS transmission pattern in Singapore reassessed by viral sequence variation analysis. *PLoS Med,* 2**,** e43.
LIVNI, G., RACHMEL, A., MAROM, D., YAARI, A., TIROSH, N. & ASHKENAZI S. 2010. Padeiatric Infect Dis J, 29, 71-73.
LJUBIN-STERNAK, S., SANTAK, M., CEPIN-BOGOVIĆ, J., BAĆE, A., VOJNOVIĆ, G., MLINARIĆ-GALINOVIĆ, G., FORCIĆ, D., DRAZENOVIĆ, V. & FALSEY, A. 2008. Detection of genetic lineages of human metapneumovirus in Croatia during the winter season 2005/2006. *J Med Virol,* 80**,** 1282-7.
LODHA, R., PURANIK, M., NATCHU, U. & KABRA, S. 2002. Recurrent pneumonia in children: clinical profile and underlying causes. *Acta Paediatr,* 91**,** 1170-3.
LOO, L., TAN, B., NG, L., TEE, N., LIN, R. & SUGRUE, R. 2007. Human metapneumovirus in children, Singapore. *Emerg Infect Dis,* 13**,** 1396-8.
LOUIE, J., HACKER, J., GONZALES, R., MARK, J., MASELLI, J., YAGI, S. & DREW, W. 2005. Characterization of viral agents causing acute respiratory infection in a San Francisco University Medical Center Clinic during the influenza season. *Clin Infect Dis,* 41**,** 822-8.
LUDEWICK, H., ABED, Y., VAN NIEKERK, N., BOIVIN, G., KLUGMAN, K. & MADHI, S. 2005. Human metapneumovirus genetic variability, South Africa. *Emerg Infect Dis,* 11**,** 1074-8.
MACKAY, I. 2008. Human rhinoviruses: the cold wars resume. *Rev. J Clin Virol,* 42**,** 297-320 .
MACKAY, I., ARDEN, K. & NITSCHE, A. 2002. Real-time PCR in virology. *Nucleic Acids Res,* 30**,** 1292-305.
MACKAY, I., BIALASIEWICZ, S., JACOB, K., MCQUEEN, E., ARDEN, K., NISSEN, M. & SLOOTS, T. 2006. Genetic diversity of human metapneumovirus over 4 consecutive years in Australia. *J Infect Dis,* 193**,** 1630-3.
MAHONY, J. 2008. Detection of respiratory viruses by molecular methods. *Clin Microbiol Rev,* 21**,** 716-47.

MALLEY, R., DEVINCENZO, J., RAMILO, O., DENNEHY, P., MEISSNER, H., GRUBER, W., SANCHEZ, P., JAFRI, H., BALSLEY, J., CARLIN, D., BUCKINGHAM, S., VERNACCHIO, L. & AMBROSINO, D. 1998. Reduction of respiratory syncytial virus (RSV) in tracheal aspirates in intubated infants by use of humanized monoclonal antibody to RSV F protein. *J Infect Dis,* 178**,** 1555-61.

MALLEY, R., VERNACCHIO, L., DEVINCENZO, J., RAMILO, O., DENNEHY, P., MEISSNER, H. & ET AL., 2000. Enzyme-linked immunosorbent assay to assess respiratory syncytial virus concentration and correlate results with inflammatory mediators in tracheal secretions. *Pediatr Infect Dis J,* 19, 1-7.

MALMSTRÖM, K., PITKÄRANTA, A., CARPEN, O., PELKONEN, A., MALMBERG, L., TURPEINEN, M., KAJOSAARI, M., SARNA, S., LINDAHL, H., HAAHTELA, T. & MÄKELÄ, M. 2006. Human rhinovirus in bronchial epithelium of infants with recurrent respiratory symptoms. *J Allergy Clin Immunol,* 118**,** 591-6.

MANOHA, C., ESPINOSA, S., AHO, S., HUET, F. & POTHIER, P. 2007. Epidemiological and clinical features of hMPV, RSV and RVs infections in young children. *J Clin Virol,* 38**,** 221-6.

MARGUET, C., LUBRANO, M., GUEUDIN, M., LE ROUX, P., DESCHILDRE, A., FORGET, C., COUDERC, L., SIRET, D., DONNOU, M., BUBENHEIM, M., VABRET, A. & FREYMUTH, F. 2009. In very young infants severity of acute bronchiolitis depends on carried viruses. *PLoS One,* 4**,** e4596.

MARTIN, E., KUYPERS, J., HEUGEL, J. & ENGLUND, J. 2008. Clinical disease and viral load in children infected with respiratory syncytial virus or human metapneumovirus. *Diagn Microbiol Infect Dis,* 62**,** 382-8.

MATHISEN, M., STRAND, T., SHARMA, B., CHANDYO, R., VALENTINER-BRANTH, P., BASNET, S., ADHIKARI, R., HVIDSTEN, D., SHRESTHA, P & SOMMERFELT, H. 2010. Clinical presentation and severity of viral community-acquired pneumonia in young Nepalese children. *PIDJ,* 29, e1-10.

MATSUZAKI, Y., ITAGAKI, T., ABIKO, C., AOKI, Y., SUTO, A. & MIZUTA, K. 2008. Clinical impact of human metapneumovirus genotypes and genotype-specific seroprevalence in Yamagata, Japan. *J Med Virol,* 80**,** 1084-9.

MCERLEAN, P., SHACKELTON, L., ANDREWS, E., WEBSTER, D., LAMBERT, S., NISSEN, M., SLOOTS, T. & MACKAY, I. 2008. Distinguishing molecular features and clinical characteristics of a putative new rhinovirus species, human rhinovirus C (HRV C). *PLoS One,* 3**,** e1847.

MCERLEAN, P., SHACKELTON, L., LAMBERT, S., NISSEN, M., SLOOTS, T. & MACKAY, I. 2007. Characterisation of a newly identified human

rhinovirus, HRV-QPM, discovered in infants with bronchiolitis. *J Clin Virol,* 39**,** 67-75.
MCINTOSH, K. 2002. Community-acquired pneumonia in children. *N Engl J Med,* 346**,** 429-37.
MEERHOFF, T., HOUBEN, M., COENJAERTS, F., KIMPEN, J., HOFLAND, R., SCHELLEVIS, F & BONT L. 2010. Detection of multiple respiratory pathogens during primary respiratory infection: nasal seab versus nasopharyngeal aspirate using real-time polymerase chain reaction. *Eur J Clin Microbiol. Infect Dis,* Jan.
MICHELOW, I., OLSEN, K., LOZANO, J., DUFFY, L., MCCRACKEN, G. & HARDY, R. 2004a. Diagnostic utility and clinical significance of naso- and oropharyngeal samples used in a PCR assay to diagnose Mycoplasma pneumoniae infection in children with community-acquired pneumonia. *J Clin Microbiol,* 42**,** 3339-41.
MICHELOW, I., OLSEN, K., LOZANO, J., ROLLINS, N., DUFFY, L., ZIEGLER, T., KAUPPILA, J., LEINONEN, M. & MCCRACKEN, G. J. 2004b. Epidemiology and clinical characteristics of community-acquired pneumonia in hospitalized children. *Pediatrics,* 113**,** 701-7.
MILLER, E., LU, X., ERDMAN, D., POEHLING, K., ZHU, Y., GRIFFIN, M., HARTERT, T., ANDERSON, L., WEINBERG, G., HALL, C., IWANE, M. & EDWARDS, K. 2007. Rhinovirus-associated hospitalizations in young children. *J Infect Dis,* 195**,** 773-81.
MIRON, D., SRUGO, I., KRA-OZ, Z. & KENESS, Y. 2010. Sole Pathogen in Acute Bronchiolitis: Is there a role for ther organisms apart from respiratory syncytial virus? Pediatric Infect Dis J, 29,e7-10.
MIYAIRI, I. & DEVINCENZO, J. 2008. Human genetic factors and respiratory syncytial virus disease severity. *Clin Microbiol Rev,* 21**,** 686-703.
MMWR, 2008. Respiratory syncytial virus activity-- United States, July 2007-December 2008. *MMWR Morb Mortal Wkly Rep,* 57**,** 1355-8.
MMWR, 2009. Hospitalized patients with novel influenza A (H1N1) virus infection - California, April-May, 2009. *MMWR Morb Mortal Wkly Rep,* 58**,** 536-41.
MOHAPATRA, S. & BOYAPALLE, S. 2008. Epidemiologic, experimental, and clinical links between respiratory syncytial virus infection and asthma. *Clin Microbiol Rev,* 21**,** 495-504.
MONTO, A. 2002. The seasonality of rhinovirus infections and its implications for clinical recognition. *Clin Ther,* 24**,** 1987-97.
MORSE, D., CARROLL, D., WEBERG, L., BORGSTROM, M., RANGER-MOORE, J. & GILLIES, R. 2005. Determining suitable internal standards for mRNA quantification of increasing cancer progression in human breast cells by real-time reverse transcriptase polymerase chain reaction. *Anal Biochem,* 342**,** 69-77.

MULHOLLAND, E., SIMOES, E., COSTALES, M., MCGRATH, E., MANALAC, E. & GOVE, S. 1992. Standardized diagnosis of pneumonia in developing countries. *Pediatr Infect Dis J,* 11**,** 77-81.
MULLER, M., RICHARDSON, S., MCGEER, A., DRESSER, L., RABOUD, J., MAZZULLI, T., LOEB, M. & LOUIE, M. 2006. Early diagnosis of SARS: lessons from the Toronto SARS outbreak. *Eur J Clin Microbiol Infect Dis,* 25**,** 230-7.
MULLINS, J., LAMONTE, A., BRESEE, J. & ANDERSON, L. 2003. Substantial variability in community respiratory syncytial virus season timing. *Pediatr Infect Dis J,* 22**,** 857-62.
MURDOCH, D. 2004. Molecular genetic methods in the diagnosis of lower respiratory tract infections. *APMIS,* 112**,** 713-27.
MUTHUKUMAR, T., DADHANIA, D., DING, R., SNOPKOWSKI, C., NAQVI, R., LEE, J., HARTONO, C., LI, B., SHARMA, V., SESHAN, S., KAPUR, S., HANCOCK, W., SCHWARTZ, J. & SUTHANTHIRAN, M. 2005. Messenger RNA for FOXP3 in the urine of renal-allograft recipients. *N Engl J Med,* 353**,** 2342-51.
NASCIMENTO-CARVALHO, C., LOPES, A., GOMES, M., MAGALHÃES, M., OLIVEIRA, J., VILAS-BOAS, A., ROCHA, H. & BENGUIGUI, Y. 2001. The burden of pneumonia among children. *J Trop Pediatr,* 47**,** 253-4.
NG, E., CHENG, P., NG, A., HOANG, T. & LIM, W. 2005. Influenza A H5N1 detection. *Emerg Infect Dis,* 11**,** 1303-5.
NIESTERS, H. 2004. Molecular and diagnostic clinical virology in real time. *Clin Microbiol Infect,* 10**,** 5-11.
NIKOLAOS, S., GEORGE, A., TELEMACHOS, T., MARIA, S., YANNIS, M. & KONSTANTINOS, M. 2000. Effect of L-carnitine supplementation on red blood cells deformability in hemodialysis patients. *Ren Fail,* 22**,** 73-80.
NOKES, J. & CANE, P. 2008. New strategies for control of respiratory syncytial virus infection. *Curr Opin Infect Dis,* 21**,** 639-43.
NUWAYSIR, E., HUANG, W., ALBERT, T., SINGH, J., NUWAYSIR, K., PITAS, A., RICHMOND, T., GORSKI, T., BERG, J., BALLIN, J., MCCORMICK, M., NORTON, J., POLLOCK, T., SUMWALT, T., BUTCHER, L., PORTER, D., MOLLA, M., HALL, C., BLATTNER, F., SUSSMAN, M., WALLACE, R., CERRINA, F. & GREEN, R. 2002. Gene expression analysis using oligonucleotide arrays produced by maskless photolithography. *Genome Res,* 12**,** 1749-55.
O'BRIEN, M., UYEKI, T., SHAY, D., THOMPSON, W., KLEINMAN, K., MCADAM, A., YU, X., PLATT, R. & LIEU, T. 2004. Incidence of outpatient visits and hospitalizations related to influenza in infants and young children. *Pediatrics,* 113**,** 585-93.
OGRA, P. 2004. Respiratory syncytial virus: the virus, the disease and the immune response. *Paediatr Respir Rev,* 5 Suppl A**,** S119-26.

OKIRO, E., NGAMA, M., BETT, A., CANE, P., MEDLEY, G. & JAMES NOKES, D. 2008. Factors associated with increased risk of progression to respiratory syncytial virus-associated pneumonia in young Kenyan children. *Trop Med Int Health,* 13**,** 914-26.

OLIVEIRA, T., FREITAS, G., RIBEIRO, L., YOKOSAWA, J., SIQUEIRA, M., PORTES, S., SILVEIRA, H., CALEGARI, T., COSTA, L., MANTESE, O. & QUEIRÓZ, D. 2008. Prevalence and clinical aspects of respiratory syncytial virus A and B groups in children seen at Hospital de Clínicas of Uberlândia, MG, Brazil. *Mem Inst Oswaldo Cruz,* 103**,** 417-22.

OLSZEWSKA, W. & OPENSHAW, P. 2009. Emerging drugs for respiratory syncytial virus infection. *Expert Opin Emerg Drugs,* 14**,** 207-17.

OMER, S., SUTANTO, A., SARWO, H., LINEHAN, M., DJELANTIK, I., MERCER, D., MONIAGA, V., MOULTON, L., WIDJAYA, A., MULJATI, P., GESSNER, B. & STEINHOFF, M. 2008. Climatic, temporal, and geographic characteristics of respiratory syncytial virus disease in a tropical island population. *Epidemiol Infect,* 136**,** 1319-27.

ONG, B., GAO, Q., PHOON, M., CHOW, V., TAN, W. & VAN BEVER, H. 2007. Identification of human metapneumovirus and Chlamydophila pneumoniae in children with asthma and wheeze in Singapore. *Singapore Med J,* 48**,** 291-3.

ORDÁS, J., BOGA, J., ALVAREZ-ARGÜELLES, M., VILLA, L., RODRÍGUEZ-DEHLI, C., DE OÑA, M., RODRÍGUEZ, J. & MELÓN, S. 2006. Role of metapneumovirus in viral respiratory infections in young children. *J Clin Microbiol,* 44**,** 2739-42.

OSTAPCHUK, M., ROBERTS, D. & HADDY, R. 2004. Community-acquired pneumonia in infants and children. *Am Fam Physician,* 70**,** 899-908.

OVERWEG, K., HERMANS, P., TRZCINSKI, K., SLUIJTER, M., DE GROOT, R. & HRYNIEWICZ, W. 1999. Multidrug-resistant Streptococcus pneumoniae in Poland: identification of emerging clones. *J Clin Microbiol,* 37**,** 1739-45.

PALKA-SANTINI, M., CLEVEN, B., EICHINGER, L., KRÖNKE, M. & KRUT, O. 2009. Large scale multiplex PCR improves pathogen detection by DNA microarrays. *BMC Microbiol,* 9**,** 1.

PANOZZO, C., FOWLKES, A. & ANDERSON, L. 2007. Variation in timing of respiratory syncytial virus outbreaks: lessons from national surveillance. *Pediatr Infect Dis J,* 26**,** S41-5.

PAPADOPOULOS, N. 2004. Do rhinoviruses cause pneumonia in children? *Paediatr Respir Rev,* 5 Suppl A**,** S191-5.

PAPADOPOULOS, N., MOUSTAKI, M., TSOLIA, M., BOSSIOS, A., ASTRA, E., PREZERAKOU, A., GOURGIOTIS, D. & KAFETZIS, D. 2002. Association of rhinovirus infection with increased disease severity in acute bronchiolitis. *Am J Respir Crit Care Med,* 165**,** 1285-9.

PARANHOS-BACCALÀ, G., KOMURIAN-PRADEL, F., RICHARD, N., VERNET, G., LINA, B. & FLORET, D. 2008. Mixed respiratory virus infections. *J Clin Virol,* 43**,** 407-10.

PARVEEN, S., BROOR, S., KAPOOR, S., FOWLER, K. & SULLENDER, W. 2006. Genetic diversity among respiratory syncytial viruses that have caused repeated infections in children from rural India. *J Med Virol,* 78**,** 659-65.

PATICK, A. 2006. Rhinovirus chemotherapy. *Antiviral Res,* 71**,** 391-6.

PELTOLA, V., WARIS, M., OSTERBACK, R., SUSI, P., HYYPIÄ, T. & RUUSKANEN, O. 2008. Clinical effects of rhinovirus infections. *J Clin Virol,* 43**,** 411-4.

PERET, T., BOIVIN, G., LI, Y., COUILLARD, M., HUMPHREY, C., OSTERHAUS, A., ERDMAN, D. & ANDERSON, L. 2002. Characterization of human metapneumoviruses isolated from patients in North America. *J Infect Dis,* 185**,** 1660-3.

PERET, T., HALL, C., HAMMOND, G., PIEDRA, P., STORCH, G., SULLENDER, W. & ET. AL. 2000. Circulation patterns of group A and B human respiratory syncytial virus genotypes in 5 communities in North America. *J Infect Dis,* 181, 1891-1896.

PERKINS, S., WEBB, D., TORRANCE, S., EL SALEEBY, C., HARRISON, L., AITKEN, J., PATEL, A. & DEVINCENZO, J. 2005. Comparison of a real-time reverse transcriptase PCR assay and a culture technique for quantitative assessment of viral load in children naturally infected with respiratory syncytial virus. *J Clin Microbiol,* 43**,** 2356-62.

PIRALLA, A., ROVIDA, F., CAMPANINI, G., ROGNONI, V., MARCHI, A., LOCATELLI, F & GERNA, G. 2009. Clinical severity and molecular typing of human rhinovirus C strains during a fall outbreak affecting hospitalized patients. *J Clin Virol,* 45, 311-317.

PITKÄRANTA, A. & HAYDEN, F. 1998. Rhinoviruses: important respiratory pathogens. *Ann Med,* 30**,** 529-37.

PORTEN, K., FAENSEN, D. & KRAUSE, G. SARS outbreak in Germany 2003: workload of local health departments and their compliance in quarantine measures--implications for outbreak modeling and surge capacity? *J Public Health Manag Pract,* 12**,** 242-7.

PULI, V. & CLARKE, S. 2002. Clinical aspects of pneumonia. *Br J Biomed Sci,* 59**,** 170-2.

RAOULT, D., FOURNIER, P. & DRANCOURT, M. 2004. What does the future hold for clinical microbiology? *Nat Rev Microbiol,* 2**,** 151-9.

RAWLINSON, W., WALIUZZAMAN, Z., CARTER, I., BELESSIS, Y., GILBERT, K. & MORTON, J. 2003. Asthma exacerbations in children associated with rhinovirus but not human metapneumovirus infection. *J Infect Dis,* 187**,** 1314-8.

REICHE, J. & SCHWEIGER, B. 2009. Genetic variability of group A human respiratory syncytial virus strains circulating in Germany from 1998 to 2007. *J Clin Microbiol,* 47**,** 1800-10.

RICHARD, N., KOMURIAN-PRADEL, F., JAVOUHEY, E., PERRET, M., RAJOHARISON, A., BAGNAUD, A., BILLAUD, G., VERNET, G., LINA, B., FLORET, D. & PARANHOS-BACCALÀ, G. 2008. The impact of dual viral infection in infants admitted to a pediatric intensive care unit associated with severe bronchiolitis. *Pediatr Infect Dis J,* 27**,** 213-7.

ROBERTSON, S., ROCA, A., ALONSO, P., SIMOES, E., KARTASASMITA, C., OLALEYE, D., ODAIBO, G., COLLINSON, M., VENTER, M., ZHU, Y. & WRIGHT, P. 2004. Respiratory syncytial virus infection: denominator-based studies in Indonesia, Mozambique, Nigeria and South Africa. *Bull World Health Organ,* 82**,** 914-22.

ROSSI, G., MEDICI, M., ARCANGELETTI, M., LANARI, M., MEROLLA, R., PAPARATTI, U., SILVESTRI, M., PISTORIO, A. & CHEZZI, C. 2007. Risk factors for severe RSV-induced lower respiratory tract infection over four consecutive epidemics. *Eur J Pediatr,* 166**,** 1267-72.

ROSSMANN, G., HE, Y. & KUHN, R.J. 2002. Picornavirus-receptor interactions. *Trends Microbiol,* 10, 324-31.

ROTH, S., JALAVA, J., RUUSKANEN, O., RUOHOLA, A. & NIKKARI, S. 2004. Use of an oligonucleotide array for laboratory diagnosis of bacteria responsible for acute upper respiratory infections. *J Clin Microbiol,* 42**,** 4268-74.

RUSSELL-TAYLOR, M. 2000. Bacterial pneumonias: management and complication. *Paediatr Respir Rev,* 1**,** 14-20.

SAIMAN, L. 2004. Microbiology of early CF lung disease. *Paediatr Respir Rev,* 5 Suppl A**,** S367-9.

SATO, M., SAITO, R., SAKAI, T., SANO, Y., NISHIKAWA, M., SASAKI, A., SHOBUGAWA, Y., GEJYO, F. & SUZUKI, H. 2005. Molecular epidemiology of respiratory syncytial virus infections among children with acute respiratory symptoms in a community over three seasons. *J Clin Microbiol,* 43**,** 36-40.

SATTAR, S., ABEBE, M., BUETI, A., JAMPANI, H., NEWMAN, J. & HUA, S. 2000. Activity of an alcohol-based hand gel against human adeno-, rhino-, and rotaviruses using the fingerpad method. *Infect Control Hosp Epidemiol,* 21**,** 516-9.

SAVOLAINEN, C., BLOMQVIST, S. & HOVI, T. 2003. Human rhinoviruses. *Paediatr Respir Rev,* 4**,** 91-8.

SAVOLAINEN, C., RÅMAN, L., ROIVAINEN, M. & HOVI, T. 2002. Human rhinovirus 87 and enterovirus 68 represent a unique serotype with rhinovirus and enterovirus features. *J Clin Microbiol,* 40**,** 4218-23.

SAVON, C., GOYENECHEA, A., VALDES, O., AGUILAR, J., GONZALEZ, G., PALERM, L., GONZALEZ, G. & BRENA P. 2006. Respiratory syncytial virus group A and B genotypes and disease severity among Cuban children. *Arc Med Res,* 37, 543-547.

SCHELTINGA, S., TEMPLETON, K., BEERSMA, M. & CLAAS, E. 2005. Diagnosis of human metapneumovirus and rhinovirus in patients with

respiratory tract infections by an internally controlled multiplex real-time RNA PCR. *J Clin Virol,* 33**,** 306-11.
SCHICKLI, J., DUBOVSKY, F. & TANG, R. 2009. Challenges in developing a pediatric RSV vaccine. *Hum Vaccin,* 5.
SCHMITTGEN, T. & ZAKRAJSEK, B. 2000. Effect of experimental treatment on housekeeping gene expression: validation by real-time, quantitative RT-PCR. *J Biochem Biophys Methods,* 46**,** 69-81.
SCHRAG, S., MCGEE, L., WHITNEY, C., BEALL, B., CRAIG, A., CHOATE, M., JORGENSEN, J., FACKLAM, R. & KLUGMAN, K. 2004. Emergence of Streptococcus pneumoniae with very-high-level resistance to penicillin. *Antimicrob Agents Chemother,* 48**,** 3016-23.
SEMPLE, M., BOOTH, J. & EBRAHIMI, B. 2007. Most human metapneumovirus and human respiratory syncytial virus in infants nasal secretions is cell free. 2007. *J Clin Virol,* 40, 241-244.
SEMPLE, M., COWELL, A., DOVE, W., GREENSILL, J., MCNAMARA, P., HALFHIDE, C., SHEARS, P., SMYTH, R. & HART, C. 2005. Dual infection of infants by human metapneumovirus and human respiratory syncytial virus is strongly associated with severe bronchiolitis. *J Infect Dis,* 191**,** 382-6.
SHEK, L. & LEE, B. 2003. Epidemiology and seasonality of respiratory tract virus infections in the tropics. *Paediatr Respir Rev,* 4**,** 105-11.
SIEZEN, C., BONT, L., HODEMAEKERS, H., ERMERS, M., DOORNBOS, G., SLOT, R., WIJMENGA, C., HOUWELINGEN, H., KIMPEN, J., KIMMAN, T., HOEBEE, B. & JANSSEN, R. 2009. Genetic Susceptibility to respiratory syncytial virus bronchiolitis in preterm children is associated with airway remodling genes and innate immune genes. *Pediatr Infect Dis J,* 28, 333-334.
SIMOES, E. 2002. Immunoprophylaxis of respiratory syncytial virus: global experience. *Respir Res,* 3 Suppl 1**,** S26-33.
SINANIOTIS, C. 2004. Viral pneumoniae in children: incidence and aetiology. *Paediatr Respir Rev,* 5 Suppl A**,** S197-200.
SINGH, V. 2005. The burden of pneumonia in children: an Asian perspective. *Paediatr Respir Rev,* 6**,** 88-93.
SLOOTS, T., MACKAY, I., BIALASIEWICZ, S., JACOB, K., MCQUEEN, E., HARNETT, G., SIEBERT, D., MASTERS, B., YOUNG, P. & NISSEN, M. 2006. Human metapneumovirus, Australia, 2001-2004. *Emerg Infect Dis,* 12**,** 1263-6.
SLOOTS, T., MACKAY, I., BIALASIEWICZ, S., JACOB, K., MCQUEEN, E., HARNETT, G., SIEBERT, D., MASTERS, B., YOUNG, P. & NISSEN, M. 2006. Human metapneumovirus, Australia, 2001-2004. *Emerg Infect Dis,* 12**,** 1263-6.
SLOOTS, T., WHILEY, D., LAMBERT, S. & NISSEN, M. 2008. Emerging respiratory agents: new viruses for old diseases? *J Clin Virol,* 42**,** 233-43.

SMYTH, A. 2002. Pneumonia due to viral and atypical organisms and their sequelae. *Br Med Bull,* 61**,** 247-62.

SOH, S., POH, C. & LIN, R. 2000. Serotype distribution and antimicrobial resistance of Streptococcus pneumoniae isolates from pediatric patients in Singapore. *Antimicrob Agents Chemother,* 44**,** 2193-6.

STENSBALLE, L., HJULER, T., ANDERSEN, A., KALTOFT, M., RAVN, H., AABY, P. & SIMOES, E. 2008. Hospitalization for respiratory syncytial virus infection and invasive pneumococcal disease in Danish children aged <2 years: a population-based cohort study. *Clin Infect Dis,* 46**,** 1165-71.

STIRK, H. & THORNTON, J. 1994. The BC loop in poliovirus coat protein VP1: an ideal acceptor site for major insertions. *Protein Eng,* 7, 47-56.

SULLENDER, W. 2000. Respiratory syncytial virus genetic and antigenic diversity. *Clin Microbiol Rev,* 13**,** 1-15, table of contents.

SUNG, R., CHAN, P., TSEN, T., LI, A., LAM, W., YEUNG, A. & NELSON, E. 2009. Identification of viral and atypical bacterial pathogens in children hospitalized with acute respiratory infections in Hong Kong by multiplex PCR assays. *J Med Virol,* 81**,** 153-9.

SUNG, R., CHAN, R., TAM, J., CHENG, A. & MURRAY, H. 1992. Epidemiology and aetiology of acute bronchiolitis in Hong Kong infants. *Epidemiol Infect,* 108**,** 147-54.

SUWANJUTHA S, SUNAKORN P, CHANTAROJANASIRI T, SIRITANTIKORN S, NAWANOPARATKUL S, RATTANADILOK B, TEEYAPAIBOONSILPA P, PREUTTHIPAN A, SAREEBUTR W, PUTHAVATHANA P. 2002. Respiratory syncytial virus-associated lower respiratory tract infection in under-5-year-old children in a rural community of central Thailand, a population-based study. *J Med Assoc Thai.* 85, S1111-1119.

TAPPAREL, C., JUNIER, T., GERLACH, D., CORDEY, S., VAN BELLE, S., PERRIN, L., ZDOBNOV, E. & KAISER, L. 2007. New complete genome sequences of human rhinoviruses shed light on their phylogeny and genomic features. *BMC Genomics,* 8**,** 224.

TEMPLETON, K., SCHELTINGA, S., VAN DEN EEDEN, W., GRAFFELMAN, A., VAN DEN BROEK, P. & CLAAS, E. 2005. Improved diagnosis of the etiology of community-acquired pneumonia with real-time polymerase chain reaction. *Clin Infect Dis,* 41**,** 345-51.

THANASUGARN, W., SAMRANSAMRUAJKIT, R., VANAPONGTIPAGORN, P., PRAPPHAL, N., VAN DEN HOOGEN, B., OSTERHAUS, A. & POOVORAWAN, Y. 2003. Human metapneumovirus infection in Thai children. *Scand J Infect Dis,* 35**,** 754-6.

THOMAS, E. & BOOK, L. 1991. Comparison of two rapid methods for detection of respiratory syncytial virus (RSV) (Testpack RSV and ortho RSV ELISA) with direct immunofluorescence and virus isolation

for the diagnosis of pediatric RSV infection. *J Clin Microbiol,* 29**,** 632-5.
TURNER, R. 2001. Ineffectiveness of intranasal zinc gluconate for prevention of experimental rhinovirus colds. *Clin Infect Dis,* 33**,** 1865-70.
TURNER, R. 2005. New considerations in the treatment and prevention of rhinovirus infections. *Pediatr Ann,* 34**,** 53-7.
TURNER, R. 2007. Rhinovirus: more than just a common cold virus. *J Infect Dis,* 195**,** 765-6.
VAN DEN HOOGEN, B., BESTEBROER, T., OSTERHAUS, A. & FOUCHIER, R. 2002. Analysis of the genomic sequence of a human metapneumovirus. *Virology,* 295**,** 119-32.
VAN DEN HOOGEN, B., DE JONG, J., GROEN, J., KUIKEN, T., DE GROOT, R., FOUCHIER, R. & OSTERHAUS, A. 2001. A newly discovered human pneumovirus isolated from young children with respiratory tract disease. *Nat Med,* 7**,** 719-24.
VAN DEN HOOGEN, B., HERFST, S., SPRONG, L., CANE, P., FORLEO-NETO, E., DE SWART, R., OSTERHAUS, A. & FOUCHIER, R. 2004. Antigenic and genetic variability of human metapneumoviruses. *Emerg Infect Dis,* 10**,** 658-66.
VAN DER HOEK, L., PYRC, K., JEBBINK, M., VERMEULEN-OOST, W., BERKHOUT, R., WOLTHERS, K., WERTHEIM-VAN DILLEN, P., KAANDORP, J., SPAARGAREN, J. & BERKHOUT, B. 2004. Identification of a new human coronavirus. *Nat Med,* 10**,** 368-73.
VAN ELDEN, L., SACHS, A., VAN LOON, A., HAARMAN, M., VAN DE VIJVER, D., KIMMAN, T., ZUITHOFF, P., SCHIPPER, P., VERHEIJ, T. & NIJHUIS, M. 2008. Enhanced severity of virus associated lower respiratory tract disease in asthma patients may not be associated with delayed viral clearance and increased viral load in the upper respiratory tract. *J Clin Virol,* 41**,** 116-21.
VAN ELDEN, L., VAN LOON, A., VAN DER BEEK, A., HENDRIKSEN, K., HOEPELMAN, A., VAN KRAAIJ, M., SCHIPPER, P. & NIJHUIS, M. 2003. Applicability of a real-time quantitative PCR assay for diagnosis of respiratory syncytial virus infection in immunocompromised adults. *J Clin Microbiol,* 41**,** 4378-81.
VAN WOENSEL, J., BOS, A., LUTTER, R., ROSSEN, J. & SCHUURMAN, R. 2006. Absence of human metapneumovirus co-infection in cases of severe respiratory syncytial virus infection. *Pediatr Pulmonol,* 41**,** 872-4.
VICENTE, D., MONTES, M., CILLA, G., PEREZ-YARZA, E. & PEREZ-TRALLERO, E. 2006. Differences in clinical severity between genotype A and genotype B human metapneumovirus infection in children. *Clin Infect Dis,* 42**,** e111-3.
VIEGAS, M. & MISTCHENKO, A. 2005. Molecular epidemiology of human respiratory syncytial virus subgroup A over a six-year period (1999-2004) in Argentina. *J Med Virol,* 77**,** 302-10.

VON WINTZINGERODE, F., BÖCKER, S., SCHLÖTELBURG, C., CHIU, N., STORM, N., JURINKE, C., CANTOR, C., GÖBEL, U. & VAN DEN BOOM, D. 2002. Base-specific fragmentation of amplified 16S rRNA genes analyzed by mass spectrometry: a tool for rapid bacterial identification. *Proc Natl Acad Sci U S A,* 99**,** 7039-44.

VORA, G., MEADOR, C., STENGER, D. & ANDREADIS, J. 2004. Nucleic acid amplification strategies for DNA microarray-based pathogen detection. *Appl Environ Microbiol,* 70**,** 3047-54.

WALSH, E., MCCONNOCHIE, K., LONG, C. & HALL, C. 1997. Severity of respiratory syncytial virus infection is related to virus strain. *J Infect Dis,* 175**,** 814-20.

WANG, D., COSCOY, L., ZYLBERBERG, M., AVILA, P., BOUSHEY, H., GANEM, D. & DERISI, J. 2002. Microarray-based detection and genotyping of viral pathogens. *Proc Natl Acad Sci U S A,* 99**,** 15687-92.

WANG, D., CUMMINS, C., BAYLISS, S., SANDERCOCK, J. & BURLS, A. 2008a. Immunoprophylaxis against respiratory syncytial virus (RSV) with palivizumab in children: a systematic review and economic evaluation. *Health Technol Assess,* 12**,** iii, ix-x, 1-86.

WANG, X., JIANG, N., FENG, X., XIE, Y., TONELLATO, P., GHOSH, S. & HESSNER, M. 2003. A novel approach for high-quality microarray processing using third-dye array visualization technology. *IEEE Trans Nanobioscience,* 2**,** 193-201.

WANG, Z., MALANOSKI, A., LIN, B., KIDD, C., LONG, N., BLANEY, K., THACH, D., TIBBETTS, C. & STENGER, D. 2008b. Resequencing microarray probe design for typing genetically diverse viruses: human rhinoviruses and enteroviruses. *BMC Genomics,* 9**,** 577.

WEBER, M., DACKOUR, R., USEN, S., SCHNEIDER, G., ADEGBOLA, R., CANE, P., JAFFAR, S., MILLIGAN, P., GREENWOOD, B., WHITTLE, H. & MULHOLLAND, E. 1998. The clinical spectrum of respiratory syncytial virus disease in The Gambia. *Pediatr Infect Dis J,* 17**,** 224-30.

WELLIVER, R. 2004. Respiratory syncytial virus infection: therapy and prevention. *Paediatr Respir Rev,* 5 Suppl A**,** S127-33.

WELLIVER, R. S. 2007. Temperature, humidity, and ultraviolet B radiation predict community respiratory syncytial virus activity. *Pediatr Infect Dis J,* 26**,** S29-35.

WHITNEY, C. & HARPER, S. 2004. Lower respiratory tract infections: prevention using vaccines. *Infect Dis Clin North Am,* 18**,** 899-917.

WILKESMANN, A., SCHILDGEN, O., EIS-HÜBINGER, A., GEIKOWSKI, T., GLATZEL, T., LENTZE, M., BODE, U. & SIMON, A. 2006. Human metapneumovirus infections cause similar symptoms and clinical severity as respiratory syncytial virus infections. *Eur J Pediatr,* 165**,** 467-75.

WILLIAMS, J., HARRIS, P., TOLLEFSON, S., HALBURNT-RUSH, L., PINGSTERHAUS, J., EDWARDS, K., WRIGHT, P. & CROWE, J. J.

2004. Human metapneumovirus and lower respiratory tract disease in otherwise healthy infants and children. *N Engl J Med,* 350**,** 443-50.
WILLIAMS, J., WANG, C., YANG, C., TOLLEFSON, S., HOUSE, F., HECK, J., CHU, M., BROWN, J., LINTAO, L., QUINTO, J., CHU, D., SPAETE, R., EDWARDS, K., WRIGHT, P. & CROWE, J. J. 2006. The role of human metapneumovirus in upper respiratory tract infections in children: a 20-year experience. *J Infect Dis,* 193**,** 387-95.
WOLF, B., REY, L., BRISSE, S., MOREIRA, L., MILATOVIC, D., FLEER, A., ROORD, J. & VERHOEF, J. 2000. Molecular epidemiology of penicillin-resistant Streptococcus pneumoniae colonizing children with community-acquired pneumonia and children attending day-care centres in Fortaleza, Brazil. *J Antimicrob Chemother,* 46**,** 757-65.
WONG, C., HENG, C., WAN YEE, L., SOH, S., KARTASASMITA, C., SIMOES, E., HIBBERD, M., SUNG, W. & MILLER, L. 2007. Optimization and clinical validation of a pathogen detection microarray. *Genome Biol,* 8**,** R93.
WOO, P., LAU, S., TSOI, H., HUANG, Y., POON, R., CHU, C., LEE, R., LUK, W., WONG, G., WONG, B., CHENG, V., TANG, B., WU, A., YUNG, R., CHEN, H., GUAN, Y., CHAN, K. & YUEN, K. 2005. Clinical and molecular epidemiological features of coronavirus HKU1-associated community-acquired pneumonia. *J Infect Dis,* 192**,** 1898-907.
WORLD HEALTH ORGANISATION (WHO).1995. Acute respiratory infections in children: case manahement in small hospitals in developing countries- a manual for physicians and other senior health workers.
WRIGHT, P., GRUBER, W., PETERS, M., REED, G., ZHU, Y., ROBINSON, F., COLEMAN-DOCKERY, S. & GRAHAM, B. 2002. Illness severity, viral shedding, and antibody responses in infants hospitalized with bronchiolitis caused by respiratory syncytial virus. *J Infect Dis,* 185**,** 1011-8.
YAN X, ZHONG W, TANG A, SCHIELKE EG, HANG W, NOLAN JP. 2005. Multiplexed flow cytometric immunoassay for influenza virus detection and differentiation. *Anal Chem.* 23, 7673-8.
YE, R., WANG, T., BEDZYK, L. & CROKER, K. 2001. Applications of DNA microarrays in microbial systems. *J Microbiol Methods,* 47**,** 257-72.
YOSHIDA, L., SUZUKI, M., YAMAMOTO, T., NGUYEN, H., NGUYEN, C., OISHI, K., VU, T., LE, T., LE, M., YANAI, H., KILGORE, P., DANG D. & ARIYOSHI, K. 2010. Viral pathogens associated with acute respiratory infections in central vietnamese children. *PIDJ*, 29, 75-77.
YUSUF, S., PIEDIMONTE, G., AUAIS, A., DEMMLER, G., KRISHNAN, S., VAN CAESEELE, P., SINGLETON, R., BROOR, S., PARVEEN, S., AVENDANO, L., PARRA, J., CHAVEZ-BUENO, S., MURGUÍA DE SIERRA, T., SIMOES, E., SHAHA, S. & WELLIVER, R. 2007. The

relationship of meteorological conditions to the epidemic activity of respiratory syncytial virus. *Epidemiol Infect,* 135**,** 1077-90.
ZALM, M., VAN EWIJK, B., WILBRINK, B., UITERWAAL, C., WOLFS, T. & VAN DER ENT, C. 2008. Respiratory Pathogens in Children with and without Respiratory Symptoms. *J Pediatr*, 154, 396-400.
ZLATEVA, K., VIJGEN, L., DEKEERSMAEKER, N., NARANJO, C. & VAN RANST, M. 2007. Subgroup prevalence and genotype circulation patterns of human respiratory syncytial virus in Belgium during ten successive epidemic seasons. *J Clin Microbiol,* 45**,** 3022-30.